EPIDEMIOLOGY AND MOLECULAR CHARACTERISATION OF *EHRLICHIA PHAGOCYTOPHILA* IN RELATION TO EMERGING EHRLICHIAE

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TABLE OF CONTENTS

DECLARATION

The work presented here is all my own except where it is indicated and the composition of the thesis was completed by myself.

María Pilar Alberdi Vélez

DEDICATION

Dedico esta tesis a mis padres, Eugenio Alberdi y Sagrario Vélez

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ABSTRACT

Ehrlichia phagocytophila (Genus *Ehrlichia*, Order *Rickettsiales*) is the pathogen responsible for Tick-borne fever, a disease of high morbidity in susceptible ruminants. These bacteria appear to be almost identical at serological and molecular level to granulocytic *Ehrlichia* species recently diagnosed in humans, dogs and horses of Europe and the United States.

A molecular description of different isolates of the pathogen is given. Samples were derived from wild and domestic vertebrate hosts from the UK, where Tick-borne fever is endemic. Molecular characterisation of a fragment from the *groE* operon gene showed higher nucleotide variation than at 16S rDNA level. Human and equine isolates from Europe differed from North American samples, which at 16S appeared to be identical. Further differences were also found between ruminant and non-ruminant granulocytic samples from Europe. Genomic analysis of less conserved genes appears necessary to provide more useful phylogenetic information that will help to clarify the relationship between closely related bacterial species.

Populations of the vector tick, *Ixodes ricinus*, were sampled and analysed to determine the prevalence of infection and clarify their role in the epidemiology of the disease. The studies indicated a low infection prevalence that seems, however, enough to maintain the pathogen in nature. The prevalence varied according to widespread sites across Britain but it was always lower than expected from information in the literature. Attempts to determine the efficiency of latent infection in sheep to transmit *Ehrlichia* to ticks were unsuccessful.

A seroepidemiological survey was undertaken using IFAT and involving samples from suspected vertebrate reservoirs of infection such as dogs, cats, horses, and deer in order to determine if those species were exposed to the pathogen and the range of hosts for the bacteria in widespread sites across Britain. The results suggested high rates of exposure in dogs from rural areas and wild roe deer. Cats showed also a high seroprevalence indicating the three vertebrate hosts were exposed to *E. phagocytophila* and mounted an immune response towards the pathogen. It

remains to be elucidated if dogs, cats and horses are accidental or competent reservoirs of infection. The presence of *E. phagocytophila* in roe deer blood and spleen samples was confirmed by PCR. Tick counts from deer legs ratified that all three stages of tick (larvae, nymphs and adults) were able to feed simultaneously upon roe deer thus supporting their role as competent reservoirs for both ticks and *E. phagocytophila* together with the serological and molecular evidence.

Cytoecetes ondiri, an African relative of *Ehrlichia phagocytophila*, was shown to cross-react in immunoblots with *E. equi* and in IFAT with *E. phagocytophila* antigens thus confirming a close antigenic relationship.

ELISA were developed using crude *E. equi* and *E. phagocytophila* as antigens and samples from several vertebrate species. The assays were validated with previous results obtained by IFAT. Data suggested that *E. equi* is a useful surrogate antigen for serologic studies until *E. phagocytophila* is routinely grown in culture. The antigenic structure of *Ehrlichia* was further characterised using mitochondria as surrogate antigens under the evidence of the phylogenetic relationship between the organelles and the bacteria. *Ehrlichia* are classified in the α-subgroup of Proteobacteria, which are believed to be the closest relatives to mitochondria. Sera from experimentally inoculated animals recognised mitochondrial antigens prior and after exposure but the responses were significantly higher after infection and challenge. Further work should be directed towards the successful cultivation of the pathogen as for HGE and *E. equi* in order to develop more reliable serological tests for *E. phagocytophila* for future epidemiological surveys. Identification of the major antigenic components of the organism will also help towards vaccine development.

ABBREVIATIONS LIST

µl	microlitre
µm	micrometer
µM	micromolar
AMA	anti-mitochondrial antibodies
AP	alkaline phosphatase
ATSA	anti-tick saliva antibodies
aoHGE	agent of human granulocytic ehrlichiosis
BCIP	5-bromo-4-chloro-3-indolyl phosphate
BIg	biotinylated immunoglobulin
BN-PAGE	blue native-polyacrylamide gel electrophoresis
bp	base pairs
BPF	Bovine Petechial Fever
BSA	bovine serum albumin
°C	degrees Celsius
cELISA	competitive enzyme linked immunosorbent assay
CF	complement fixation
CI	confidence intervals
CIA	counter-immunoelectrophoresis
ConA	Concanavalin A
CTVM	Centre for Tropical Veterinary Medicine
dH_2O	distilled water
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
ECF	East Coast Fever
EDTA	ethylene-diamine tetra-acetic acid
Ehr/8	*Ehrlichia phagocytophila* Ehr/8 isolate
FG	*Ehrlichia phagocytophila* Feral Goat isolate
FITC	fluorescein isothyacianate conjugate
GIS	geographic information systems
h	hour
HBSS	Hanks balanced salt solution
HGE	human granulocytic ehrlichiosis
HME	human monocytic ehrlichiosis
HRP	horseradish peroxidase
HSP	heat shock protein
iELISA	indirect enzyme linked immunosorbent assay
IFAT	indirect fluorescent antibody test
IgG	immunoglobulin gamma
IgM	immunoglobulin M
kDa	kilodalton
MAb	monoclonal antibodies
mg	milligram

min	minutes
ml	millilitre
mM	millimolar
NBT	nitroblue tetrazolium salt
NH_4Cl	ammonium chloride
nm	nanometer
NOMA	normally occurring mitochondrial antibodies
OD	optical density
O/N	overnight
ORF	open reading frame
OS	*Ehrlichia phagocytophila* Old Sourhope isolate
PBC	primary biliary cirrhosis
PBS	phosphate buffered saline
PBST	phosphate buffered saline containing Tween 20
PCR	polymerase chain reaction
PHA	phytohaemaglutinin
PWM	pokeweed mitogen
rDNA	ribosomal deoxyribonucleic acid
rRNA	ribosomal ribonucleic acid
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
RT	room temperature
SAC	Scottish agricultural college
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SIP	stock isotonic Percoll
TBE	tris-borate EDTA buffer
TBS	tris buffered saline
TBF	Tick-borne fever
TEMED	N, N, N', N'- Tetramethylethylenediamine
TMB	tetramethyl benzidine
Tween 20	polyoxyethylene sorbitan monolaurate
UK	United Kingdom
US	United States
UV	ultraviolet light
VIC	veterinary investigation centre
v/v	volume per volume
w/v	weight per volume

CHAPTER ONE, INTRODUCTION TO THE RATIONALE OF THIS STUDY

Ehrlichia (Cytoecetes) phagocytophila is an obligate intracellular bacterium, which causes Tick-borne fever (TBF) in sheep and pasture fever in cattle and is transmitted by *Ixodes ricinus* ticks. The organism belongs to the order *Rickettsiales*, which includes many pathogens of medical and veterinary importance such as *E. chaffeensis*, *E. canis* or *Cowdria ruminantium*. *Ehrlichia phagocytophila* is included within a genogroup that also contains *E. equi*, a pathogen for horses, and the agent of human granulocytic ehrlichiosis (HGE), recently isolated from humans, dogs and horses from North America and Europe. Genetic and serologic evidence suggest these novel granulocytic *Ehrlichia* are strain variants of the same species. Strain variation has been also found between different British isolates of *E. phagocytophila* in ruminants at serological level.

Ehrlichia phagocytophila targets cells of the haematopoietic line, mainly neutrophils and eosinophils. The bacteria invade the cells and multiply by binary fission forming typical morulae within the cytoplasm, which are diagnostic. The pathogen induces a disease characterised by high fever and severe leucopenia that may facilitate concurrent infections including tick pyaemia, listeriosis, and louping-ill. Young lambs appear to be more resistant than adults to signs of clinical infection although they undergo febrile symptoms and parasitaemia.

The bacteria may survive for months in sheep, it can be detected in blood smears after splenectomy at 48 weeks or even two years after primary infection. It is believed that the organism persists in blood, mesenteric glands, spleen and central nervous system. High levels of IgM are detected in carrier sheep indicating continuous exposure of the immune system with the pathogen. *Ehrlichia phagocytophila* does not survive in cattle as long as in sheep suggesting they are not efficient reservoirs of infection. Sheep and deer are considered the main competent reservoirs for *Ehrlichia* and wild rodents have been incriminated in the transmission of granulocytic ehrlichiosis in North America and Europe.

Ixodes ricinus hard-bodied ticks are also vectors for *Borrelia burgdorferi*, *Babesia microti* and louping-ill virus, which are all zoonotic organisms. *Ixodes* are three host ticks, they feed on a wide range of vertebrate hosts thus enabling them to acquire multiple pathogens. Concurrent infections have been found in a single tick

and there is serologic evidence of exposure to two or more pathogens in humans and animals.

Ehrlichia phagocytophila is a fastidious pathogen that has not been cultivated in vitro. The diagnosis thus has been hindered and it is based on the detection of the pathogen in peripheral blood during patent parasitaemia by means of microscopic examination of blood smears or PCR. Exposure can be determined by the detection of specific antibodies towards the bacteria by counter immunoelectrophoresis (CIE) or IFAT, results can be confirmed with immunoblots.

Under laboratory conditions, *E. phagocytophila* infection appears to induce a mild condition in sheep that resolves by itself without the need of antibiotic treatment. However, in the field the immunosuppression associated with the disease may predispose to secondary infections such as staphylococcal pyaemia or aggravate concurrent diseases leading to a serious condition that can be even fatal. Abortion storms, with as many as 91% naive animals involved, are common after the introduction of pregnant ewes to tick infested pastures but the main economic impact of TBF in the UK is considered to be through the 300000 cases per year of the associated tick pyaemia in lambs leading to ill growth and loss of productivity.

Previous research in CTVM led by Dr. Gordon Scott has been directed towards isolation and cross-immunity experiments with different strains of *E. phagocytophila* obtained from domestic and wild ruminants. Many isolates were thus available for study using newly developed techniques such as PCR and sequencing to further clarify their taxonomic relationship with the almost identical organisms recently isolated from humans, dogs and horses.

Ehrlichia phagocytophila is also closely related to *Cowdria ruminantium*, a tick-borne rickettsia that causes a serious disease in non-indigenous breeds of cattle and goats in Sub-Saharan Africa and the Caribbean islands. The diagnosis of this pathogen has been hampered by the serologic cross-reactions that appear to occur between *Ehrlichia* species that are also prevalent in those tropical countries. Little is known about the presence of granulocytic *Ehrlichia* in areas where *C. ruminantium* is

endemic but it has been identified in the North of Africa and antibodies cross-reacting to *C. ruminantium* have been also found in dogs.

Studies on *E. phagocytophila* will help to understand the fundamental aspects of the pathogenesis and immunogenesis of animal tick-borne rickettsial infections of temperate and tropical countries. Better disease control and management procedures may thus be developed. The available information concerning *E. phagocytophila* and the disease caused by the bacteria suggest the need for further studies on hosts associations, epidemiology and taxonomy of granulocytic *Ehrlichia*, which were the main aims of this study.

CHAPTER TWO, LITERATURE REVIEW

2.1 Aetiology

2.1.1 Taxonomy

The genus *Ehrlichia* (Tribe *Ehrlichieae*, Order *Rickettsiales*) comprises many closely related bacteria, which are known veterinary and human pathogens (Table 2.1). All these organisms are Gram (-) intracellular bacteria residing as obligate parasites in phagosomes within cells of the haematopoietic line in vertebrate hosts, appearing as compact morulae (Rikihisa, 1991).

Ehrlichia phagocytophila is the pathogen associated with Tick-borne fever (TBF) in European sheep and cattle (MacLeod and Gordon, 1932; Hudson, 1950; Juste *et al.*, 1989). It was first described in Scotland by Gordon *et al.* in 1932 inside the cytoplasm of neutrophils of febrile sheep that appeared immune to louping-ill. *Ehrlichia equi* is the organism responsible for equine ehrlichiosis in North America, first diagnosed by Gribble (1969). Stannard *et al.* (1969) observed similarities between equine ehrlichiosis and TBF in sheep and goats suggesting they were strain variants of the same species. There is little evidence of the presence of *E. equi* in Europe. Sporadic cases of granulocytic ehrlichiosis in horses of Europe have been identified (McNamee *et al.*, 1989) associated with an unclassified *Ehrlichia*. Human granulocytic ehrlichiosis (HGE) was first identified in the upper Midwest United States in 1990 then reported by Bakken *et al.* in 1994. In 1938 Tyzzer described a pathogen, *Cytoecetes microti* (new genus, new species) isolated naturally from the field vole (*Microtus pennsylvanicus*) that stained dull blue or purplish by Giemsa. It appeared to invade polymorphonuclear neutrophils and eosinophils of experimental field voles, white-footed mice (*Peromyscus leucopus*), the desert species *Peromyscus eremicus*, the common laboratory mouse and white rat. Guinea pigs, domestic rabbits, young chickens and a kitten were resistant to the infection. Little attention has been directed towards the study of this pathogen although for its morphology and cell preference within the host it is believed to belong to the *E. phagocytophila* genogroup and this indicates that a granulocytic *Ehrlichia* naturally infects rodents. Moreover, rodent reservoirs appeared to carry the infection at least 12 years before

the first cases of human granulocytic ehrlichiosis were reported in the US (Bunnell *et al.*, 1998).

The first form of human monocytic ehrlichiosis, sennetsu fever due to *E. sennetsu*, was described in Japan in 1954 (Fukuda *et al.*). *Ehrlichia risticii* is another monocytic pathogen which induces Potomac Horse Fever in the US and Europe (Rikihisa, 1991). Maeda *et al.* (1987) described the first cases of human monocytic ehrlichiosis (HME) in the US and in 1991, Anderson *et al.* defined *Ehrlichia chaffeensis* as the etiologic agent of the disease. Another monocytic *Ehrlichia*, *E. canis*, is the causative agent of tropical canine pancytopenia (TCP), first described by Donatien and Lestoquard (1935). An atypical and milder *E. canis* infection was found to occur in dogs invading granulocytes instead of monocytes (Ewing *et al.*, 1971). A new species of the genus *Ehrlichia*, *E. ewingii*, was associated with this atypical *E. canis*-like infection using phenotypic and genetic evidence. *Ehrlichia ewingii* appeared to be closely related but different from *E. chaffeensis* and *E. canis* (98 and 98.1% similarity respectively) at 16S rRNA gene level (Anderson *et al.*,1992). Another pathogen for rodents was isolated in 1993 from the spleen of a wild mouse. It was identified as a member of the genus *Ehrlichia* on the basis of morphological and antigenic comparisons (Kawahara *et al.*, 1993), then classified as a new species, *E. muris*, which appears closely related to *E. chaffeensis* (97.9% identical at 16S rDNA level) and *E. canis* (Wen *et al.*, 1995). Although both *E. chaffeensis* and *E. canis* are pathogenic for dogs, *E. muris* experimental infection does not induce disease or humoral antibody responses in dogs (Wen *et al.*, 1995).

Neorickettsia helminthoeca causes salmon poisoning disease in dogs and is closely related at 16S rDNA sequence level to *E. sennetsu* and *E. risticii* (Pretzman *et al.*, 1995). *Ehrlichia platys* infects canine thrombocytes but it does not appear to cause clinical illness (McDade, 1990). *Ehrlichia ovina* and *E. bovis*, monocytic pathogens for ruminants, are two species of *Ehrlichia* described in Africa of uncertain taxonomic status and scarce details regarding their epidemiology (Ristic and Huxsoll, 1984).

The classification of species among the genus *Ehrlichia* appears confusing as shown in Fig. 2.1. Three main clusters can be identified as shown in Table 2.1. Each

cluster is closely associated with a non-*Ehrlichia* bacterial genus. *Wolbachia pipientis* is an insect-borne intracellular endosymbiont closely related to tick-borne members of the genus. *Anaplasma marginale* is included within the *E. phagocytophila* genogroup and *C. ruminantium* within the *E. canis* cluster. In addition, serologic cross-reactions have been found to occur between *Ehrlichia* species (Jongejan *et al.*, 1989; Rikihisa, 1991; Dumler *et al.*, 1995) and with the closely related bacteria *Anaplasma* and *Cowdria ruminantium* (Logan *et al.*, 1986; Jongejan *et al.*, 1989; Kelly *et al.*, 1994). Non-specific titres occur however at low level, within the range of 1/10 to 1/40. In addition, bacterial infections frequently generate cross-reactive antibodies to heat shock proteins (Scorpio *et al.*, 1994), with typical molecular size range of 58 to 75 kDa in immunoblots (Dumler *et al.*, 1995). Samples containing antibodies to *E. equi* and *E. phagocytophila* appear to cross-react with *C. ruminantium* antigens only in immunofluorescent tests but not in immunoblots (Logan *et al.*, 1986; Jongejan *et al.*, 1989). Classification has been based hitherto on morphology, vertebrate and invertebrate hosts, cell tropism, clinical differences, geographical location and serologic cross-reactions (Ristic and Huxsoll, 1984). Several authors have suggested the reclassification of the species of the *E. sennetsu* genogroup, which includes *E. sennetsu*, *E. risticii* and *Neorickettsia helminthoeca*, in a different genus separated from *Ehrlichia* because of their unique antigenic and phenotypic characteristics, and genetic relatedness (Dumler *et al.*, 1995; Pretzman *et al.*, 1995). For example, none of the latter three species produces dense morulae during intracellular infection, which is characteristic for the rest of the species of the genus *Ehrlichia*. In addition, it is possible that bacteria in the *E. sennetsu* genogroup are all transmitted by flukes, like *N. helminthoeca*, associated with fish or snail as intermediate hosts. *Ehrlichia risticii* DNA has been recently amplified from trematode cercaria lysates released by field collected aquatic snails of areas where Potomac Horse Fever is enzootic (Reubel *et al.*, 1998).

The *Ehrlichia phagocytophila* cluster or genogroup (Fig 2.1) includes *E. equi* and a recently discovered organism, the agent of human granulocytic ehrlichiosis (HGE), that appears to affect humans, dogs, and horses in Europe and the United States (Chen *et al.*, 1994; Johansson *et al.*, 1995; Petrovec *et al.*, 1997). The newly

discovered pathogen has been hitherto identified as HGE or aoHGE (agent of human granulocytic ehrlichiosis). Some authors have suggested the description of HGE with the name '*Ehrlichia microti* Tyzzer' for its similarities to the pathogen isolated from field voles in 1938 by Tyzzer (Telford *et al.*, 1996). *Ehrlichia phagocytophila* and *E. equi* are 99.9-99.8% identical respectively to the HGE agent and isolates from cases of granulocytic ehrlichiosis in Swedish dogs and horses at 16S rRNA gene level (Chen *et al.*, 1994; Johansson *et al.*, 1995). They differ only in 2 and 3 nucleotide positions from the aoHGE. It remains to be determined if the three agents are different species or strains of the same species with tropism for different hosts. Molecular evidence based on the almost identical 16S rDNA sequence for the three pathogens suggest they belong to the same species. Serologic studies have also revealed cross-reactions between them (Johansson *et al.*, 1995; Dumler *et al.*, 1995; Greig *et al.*, 1996; Madigan *et al.*, 1996). However, genetic polymorphism of *E. equi* has been observed (Goodman *et al.*, 1996).

Table 2.1 Pathogenic *Ehrlichia* species included in the three main genogroups based on Anderson *et al.* (1991) and Chen *et al.* (1994)

Genogroup or cluster	Species	Vertebrate host	Major target cell	Vector
E. phagocytophila	*E. phagocytophila*	Ruminants	Granulocytes	*Ixodes ricinus*
	E. equi	Horses	Granulocytes	*I. dammini, I. pacificus*
	HGE agent	Humans	Granulocytes	*Ixodes* spp.
	E. platys	Dogs	Platelets	?
	Anaplasma marginale	Cattle	Monocytes	*Boophilus* spp.
E. canis	*E. canis*	Dogs	Monocytes	*Rhipicephalus* spp.
	E. chaffeensis	Humans, deer	Monocytes	*Amblyomma* and *Dermacentor* spp.
	E. ewingii	Dogs	Granulocytes	*Amblyomma* spp.
	E. muris	Rodents	Monocytes	?
	C. ruminantium	Ruminants	Endothelial cells	*Amblyomma* spp.
E. sennetsu	*E. sennetsu*	Humans	Monocytes	Ingestion of raw fish
	E. risticii	Horses	Monocytes	?
	*N. helminthoeca**	Dogs	Macrophages	Trematodes

* *Neorickettsia helminthoeca*

? Unknown

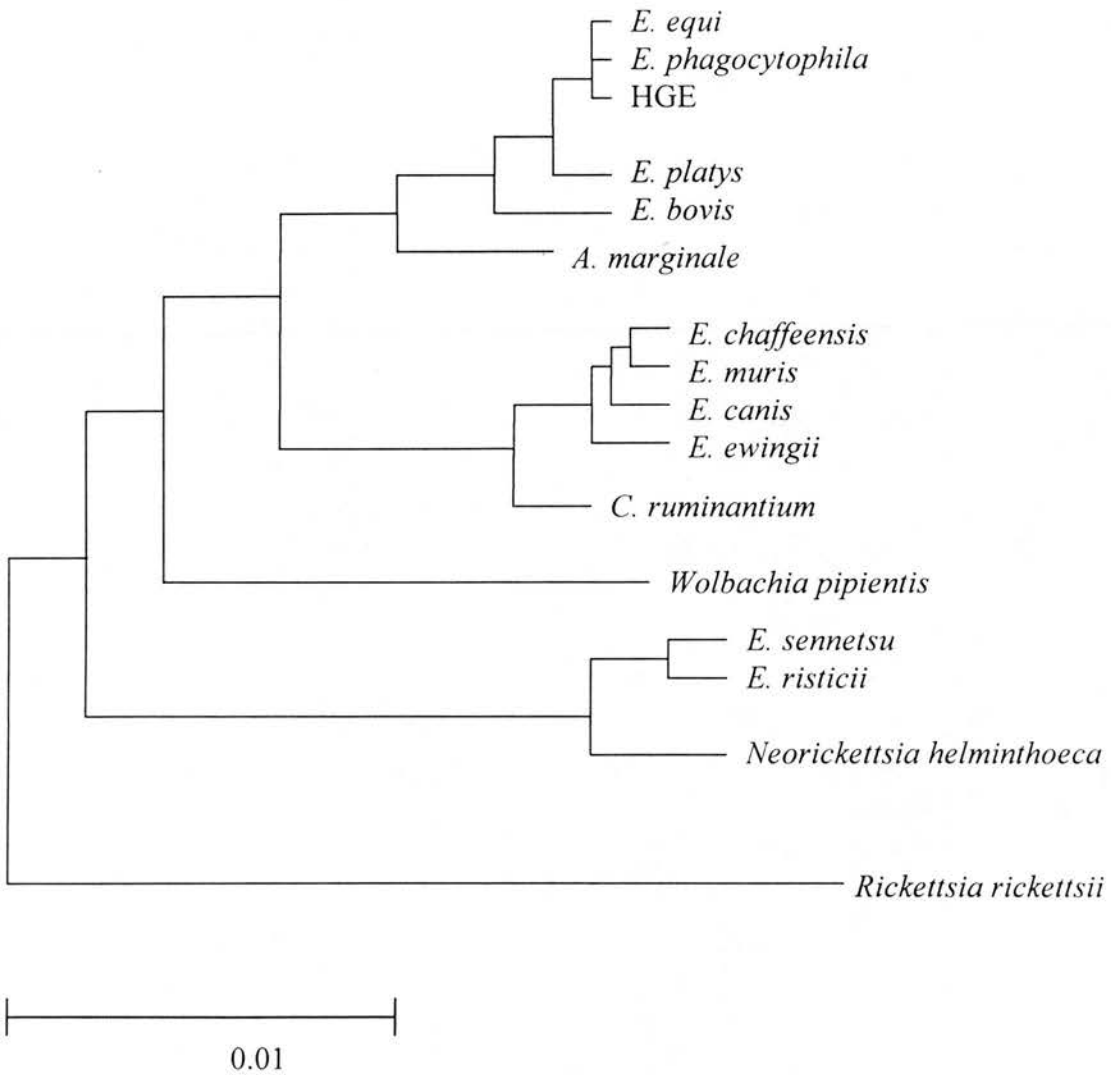


Fig 2.1 Dendrogram representing the phylogenetic relationships of bacteria of the genus *Ehrlichia* (Order *Rickettsiales*) and closely related species based on 16S rDNA sequence similarity (adapted from Walker and Dumler, 1996)

2.1.2 Life cycle

Little is known about the life history of *E. phagocytophila* within the tick through histological examination of the vector at different periods after infection. It is believed that maturation or processing of the bacteria occurs within the tick during the first stages of feeding on the host which allows the organism to become infective. Preparations from unfed ticks infected with *E. phagocytophila* were non-infective for sheep whereas those derived from partially fed ticks reproduced the infection in sheep (MacLeod, 1936). Partial blood meals in *Rhipicephalus sanguineus* nymphs, infected as larvae with *E. canis,* were also necessary to cause ehrlichiosis in dogs (Smith *et al.,* 1976). *Ehrlichia equi* cultivated in *Ixodes scapularis* tick cell line presented electro-lucent and dense forms, the latter appearing primarily in heavily infected cultures and being highly pathogenic for experimental horses (Munderloh *et al.,* 1996b). Electro-lucent and dense forms have been also found in midgut epithelium and salivary glands, respectively, of *Rhipicephalus sanguineus* ticks infected with *E. canis* (Smith *et al.,* 1976) suggesting dense forms represent the mammalian infectious stage of ehrlichiae. Life cycle within the tick in other rickettsial species such as *Anaplasma*, *Cowdria* or *Rickettsia rickettsii* are better studied. In general, these pathogens infect the gut, then the haemocytes, then the salivary glands, undergoing a simple developmental cycle involving invasion, growth and division by binary fission. *Cowdria ruminantium* reticulated forms (which are predominant) and electron-dense forms of variable size are usually found within *Amblyomma* ticks where they appear to multiply in the midgut epithelium and salivary glands (Kocan and Bezuidenhout, 1987). *Anaplasma marginale*, the primary cause of anaplasmosis in livestock, undergoes a complex cycle of development in *Dermacentor andersoni* ticks showing a wide range of forms ranging from small particles, electron-dense, large reticulated, reticulated and condensing forms (Kocan, 1986). Neither *Cowdria* nor *Anaplasma* normally invade the ovaries thus transovarial transmission is unimportant. In contrast, *Rickettsia rickettsii*, causative agent of the zoonotic Rocky Mountain Spotted Fever in the US, may infect up to 100% of the oocytes, although transovarial transmission usually ranges from 30% to 50%.

Dermacentor variabilis ticks develop a systemic infection with the rickettsia, which invades most body tissues through the haemolymph (Sonenshine, 1993).

The life cycle of *Ehrlichia phagocytophila* involves infection of the tick after the organism is ingested with the blood meal from the infected vertebrate host. The pathogen then migrates from the gut to the salivary glands. The bacteria are inoculated with tick saliva into a new host when the tick feeds in the next instar. *Ehrlichia phagocytophila* life cycle within the vertebrate host shows similarities with that of *Chlamydia psitacci* although it appears to be simpler (Woldehiwet and Scott, 1982c). *Cowdria ruminantium* grown in bovine umbilical endothelial cells culture also has developmental stages that resemble those of chlamydial species as observed by electron microscopy (Jongejan *et al.,* 1991). Despite similarities in developmental stages there is no significant phylogenetic relationship between *Ehrlichia* and *Chlamydia* species (Van Vliet *et al.,* 1992). After inoculation in the vertebrate host, particles are engulfed by phagocytosis by neutrophils, eosinophils, and monocytes. Membrane lined intracytoplasmic vacuoles, which do not fuse with lysosomes, are formed around the organisms which enlarge and multiply by binary fission. *Chlamydia* spp. are the only known bacteria that can enter the host cell by clathrin-mediated endocytosis, surface components inhibiting fusion of the phagosome with lysosomes (Moulder, 1985). This mechanism has not been demonstrated for ehrlichiae. New particles of *Ehrlichia* emerge by unknown mechanisms to infect more cells in the vertebrate host which are mainly peripheral blood neutrophils where they develop to form the characteristic morulae. As many as 95% of granulocytes may appear infected in blood smears at the height of fever (Foggie, 1951) but the parasitaemias are usually lower showing a peak of 40-50% of infection in neutrophils. The pathogen has also been found in the spleen and several tissues but located within macrophages (Campbell *et al.,* 1994). Extracellular bodies have not been observed in blood films but both plasma and serum are believed to be infective (Foggie, 1951). His experiments showed that plasma from infected sheep induced disease in susceptible animals at day 5 after experimental inoculation.

Experimentally, intravenous inoculations of *E. phagocytophila* or exposure to infected ticks are able to reproduce the disease, subcutaneous inoculation inducing

only local lesions (Taylor *et al.*, 1941; Brodie *et al.*, 1986). Transplacental transmission from the dam to the calves has been recently demonstrated (Pusterla *et al.*, 1997a). Infected neutrophils can be observed in lactating animals, however milk and colostrum do not appear to be infective for the young. Oral transmission to calves was possible, however, when adding infective blood to their meal (Pusterla *et al.*, 1998a).

2.1.3 Morphology

Ehrlichia are pleomorphic rickettsia forming clusters in the cytoplasm of granulocytes (Fig 2.2). With Romanovsky stain they show a grey-blue colour, sometimes purple. Foggie (1951) described irregular masses of rod-shaped, round or oval clusters of bodies, round or oval morulae or very small masses at the height of reaction ranging in size from 0.5 to 3.5 μm in diameter when using McNeal's modification of Leishman's stain. Tyzzer (1938) and Telford *et al.* (1996) observed a variety of *Ehrlichia* forms in mice granulocytes ranging from small rings to doublets and classical morulae suggesting that a cycle of replication occurred.

For its similarity to rickettsial organisms in morphology, intracellular location, and association with an arthropod vector, Foggie (1951) suggested that *E. phagocytophila* should be renamed *Rickettsia phagocytophila*. However, biological differences have been found and 16S rDNA sequence analysis has confirmed the divergence between the genera *Rickettsia* and *Ehrlichia* (Anderson *et al.*, 1991).

Ultrastructural examination at necropsy of human spleen tissues infected with the HGE agent revealed the characteristic morphology of *Ehrlichia*. The pathogen appeared in clusters of small, pleomorphic cocci within cytoplasmic membrane bound vacuoles in polymorphonuclear leukocytes (Chen *et al.*, 1994).

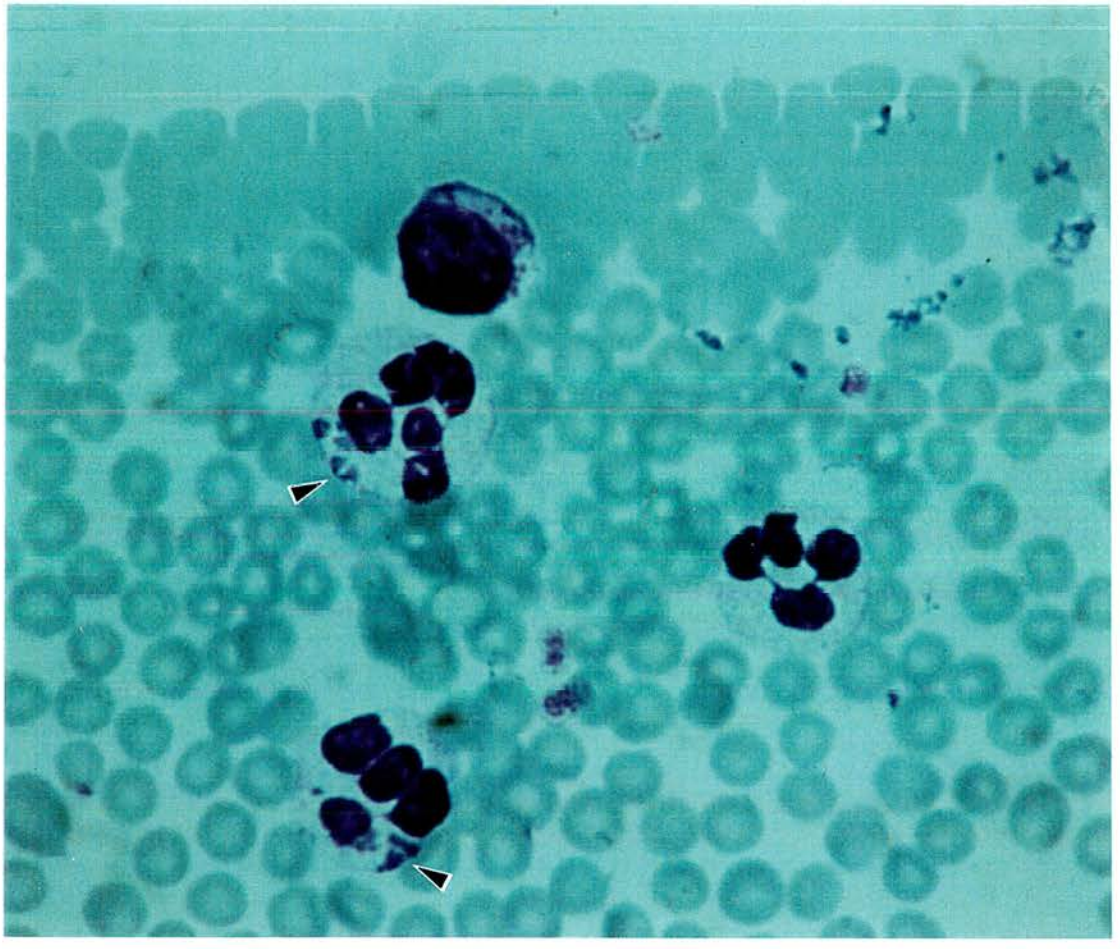


Fig 2.2 Giemsa stained blood smear showing two neutrophils infected with *E. phagocytophila* with the characteristic intracytoplasmic morulae (arrows). An uninfected neutrophil is shown at the right for comparison (x1000)

2.1.4 Strain variation: Antigenic and molecular differences

There are many strains of *E. phagocytophila* that appear to vary in virulence and antigenicity (Foggie, 1951). *Ehrlichia phagocytophila* immunological and strain differences have been based on cross-immunity trials but they have given contradictory results. For instance, Tuomi (1967b) showed that different strains did not give cross-protection but Foster and Cameron (1970a) found two immunologically identical strains isolated in different locations. Foggie and Allison (1960) claimed that bovine strains did not protect sheep from challenge with ovine strains and Tuomi (1967a) also indicated the existence of differences in virulence depending on the bovine or ovine origin of the isolates. Foggie and Allison (1960) claimed that cattle-adapted strains of the organism existed from the evidence of the distribution of the disease in tick-infested pasture where, in the absence of sheep, cattle were the main host for *I. ricinus*. Bovine strains appeared to have low infectivity for sheep. As for sheep, only bought-in animals were affected, home stock was immune because they were probably infected as calves when resistant to the clinical signs of the disease and further attacks. Woldehiwet and Scott (1982b) were able to differentiate between three strains derived from sheep, Old Sourhope (OS), Sourhope R153 (SR153) and Lephinmore-1 (L-1), using a complement fixation test. Higher titres were obtained when using homologous strains but differences were found between L-1 and the two other strains, SR153 and OS, that also appeared to be dominant over L-1. Those studies indicated that although they shared common antigens, differences were quantifiable between homologous and heterologous strains. Experiments performed by Dr. Gordon Scott in the CTVM with several *E. phagocytophila* isolates confirmed the strain antigenic diversity (Scott, CTVM Annual Reports). Antigenic variation has been observed also in different isolates of the HGE agent in North America (Asanovich *et al.*, 1997).

Molecular methods have proved to be useful tools to determine differences between strains. 16S rRNA is a well conserved gene within eubacteria which has been extensively used as a molecular clock for phylogeny studies (Wilson *et al.*, 1990; Weisburg *et al.*, 1991). Specific primers for 16S rRNA gene of *E. phagocytophila* genogroup have been designed (Dumler *et al.*,1997; Munderloh *et*

al., 1996b; Barlough *et al.*, 1996). The sequence homology within the cluster is very high, they appear almost 100% identical in a fragment of approximately 1427-bp. Further differences between granulocytic isolates might be expected when analysing less conserved genes showing more nucleotide divergences and therefore more useful phylogenetic information. An immunoreactive heat shock protein (HSP) encoded by *groE* operon has been studied in bacteria and several rickettsial organisms including *Ehrlichia chaffeensis* (Sumner *et al.*, 1993), *Cowdria ruminantium* (Lally *et al.* 1995), and granulocytic *Ehrlichia* (Sumner *et al.*, 1997), which are homologous to the *Escherichia coli groEL* protein. Although it is also conserved between species this gene shows more nucleotide changes than 16S (Kolbert *et al.*, 1997; Sumner *et al.*, 1997; Petrovec *et al.*, 1999), and thereby increased possibilities of studying strain variation. Furthermore, specific probes for the intergenic spacer of the *groESL* operon, a non-coding sequence, can be designed. That region appears to be highly variable in length and nucleotide sequences for *Cowdria ruminantium*, *E. canis* and *E. chaffeensis* (Sumner *et al.*, 1997) although less variation was found for granulocytic species. Citrate synthase gene has been used for phylogenetic studies among *Rickettsia* and *Bartonella* (Regnery *et al.*, 1991) showing also more sequence variation and more useful information for the examination of closely related organisms. The combination of two or more genes appears essential for the study of strain diversity within species or closely related bacterial species.

Genetic evidence suggests members of the *E. phagocytophila* genogroup are conspecific because of the sequence homology at 16S rDNA level and because they share significant antigenicity (Chen *et al.*, 1994; Johansson *et al.*, 1995, Dumler *et al.*, 1995; Goodman *et al.*, 1996). However, further differences in nucleotide sequence have been found in *groESL* gene (Sumner *et al.*, 1997). In addition, the three species also differ in host mammalian susceptibility and geographical localisation suggesting that they should be kept as separate species. It is unknown if the recently diagnosed dog and horse cases occur with infection by ruminant types or if they are biotypes with particular pathogenicity. There is evidence that HGE can naturally and experimentally infect sheep (Fish *et al.*, 1997) but nothing is known about the ability of ovine strains to induce disease in humans. Horses experimentally

inoculated with HGE showed clinical signs compatible with *E. equi* infection and appeared resistant to the challenge with *E. equi* (Barlough *et al.*, 1995). Experimental inoculation of *E. equi* into calves, ewes, lambs and goats did not reproduce the disease although occasionally morulae were found inside peripheral neutrophils of goats and lambs (Stannard *et al.*, 1969). Telford *et al.* (1996) successfully maintained a human strain in mice and a 45-kDa recombinant fusion protein derived from infected human blood is known to induce strong antibody responses in experimentally inoculated mice to two different strains of HGE (Kolbert *et al.*, 1997) indicating a high degree of protein conservation. Further bacterial genomic analysis appears necessary to either confirm the phylogenetic relationship and/or to reclassify the granulocytic isolates and to determine if the human pathogens belong or not to the same species as the bacteria derived from domestic and wild animals.

2.2 Epidemiology

2.2.1 Geographical distribution of granulocytic *Ehrlichia*

Granulocytic *Ehrlichia* are transmitted by ticks of the genus *Ixodes* (Fig 2.3) and their distribution therefore falls within the range of these ticks (Fig 2.4). The genus *Ixodes* is widely distributed across temperate, sub-tropical and tropical areas of the world. Ticks of the species *I. ricinus* are found throughout Europe usually associated with mild temperatures and high humidity. They can be found between 39° to 65°N latitude and as far as 60°E longitude, east of the Caspian Sea (Gray, 1991). Low densities of this species also occur in forested areas of North Africa on the northern slopes of the Atlas Mountains and Levant countries of the eastern Mediterranean. *Ixodes ricinus* and *I. persulcatus* (the species present in Eastern Europe) are known vectors for *E. phagocytophila* (MacLeod and Gordon, 1933; MacLeod 1936), *Borrelia burgdorferi* causing Lyme borreliosis, *Babesia* species (Magnarelli *et al.*, 1987; Piesman *et al.*, 1987) and flaviviruses such as louping-ill and tick-borne encephalitis viruses (MacLeod and Gordon, 1932; Hudson *et al.*, 1995; Labuda *et al.*, 1993). *Ixodes dammini* (*I. scapularis*) and *I. pacificus* are found in North America associated with Lyme disease, babesiosis (Piesman *et al.*, 1987),

equine ehrlichiosis (Richter *et al.*, 1996) and the HGE agent (Pancholi *et al.*, 1995). Oliver *et al.* (1993) demonstrated conspecificity of *I. dammini* and *I. scapularis* ticks that showed variations due to their different habitats. Despite the presence of *E. equi* and HGE affecting humans, dogs and horses, there is no evidence of infection with granulocytic *Ehrlichia* in ruminants of North America.

Ehrlichia phagocytophila was observed for the first time in Spain in 1986 by Juste *et al.* when performing experimental inoculations with blood parasites in sheep. The disease was later on diagnosed in cattle of the same area in the North of Spain (Juste *et al.*, 1989). It is believed that *E. phagocytophila* pathogens are widely distributed across the Basque Country where *I. ricinus* ticks are abundant (Barral *et al.*, 1993). The bacteria have been sporadically detected in blood from goats of Extremadura, which is located in the South West of Spain (Habela *et al.*, 1991). It is likely that the pathogen remains undiagnosed in many other areas of the country associated with suitable habitats for the tick vector.

In the UK, TBF appears prevalent over all the country of rough grazings where ticks and sheep co-exist and its distribution follows louping-ill (MacLeod, 1936). *Ixodes ricinus* ticks need a combination of high humidity and maintenance hosts for adults. These conditions are found in rough pastures for sheep or cattle, particularly in the west of Britain, which is wetter and less fertile. The presence of red deer (*Cervus elaphus*) will increase tick numbers. Conditions for maintenance of tick populations are also met in forests (natural deciduous or plantation conifers) which have populations of deer, most commonly roe deer (*Capreolus capreolus*). These are found all over Britain, with high tick populations in some eastern areas with low rainfall such at Thetford forest in East Anglia

HGE has been extensively diagnosed in several countries of Europe such as Switzerland (Brouqui *et al.*, 1995, Pusterla *et al.*, 1998b), the UK (Sumption *et al.*, 1995), Sweden (Dumler *et al.*, 1997), Norway (Bakken *et al.*, 1997), Slovenia (Petrovec *et al.*, 1997) and Bulgaria recently (Christova and Dumler, 1999).

Little is known about the presence of granulocytic *Ehrlichia* in the tropics. It is believed that *E. phagocytophila* might be present in Africa (Rikihisa, 1991). A

closely related species, *Cowdria ruminantium*, shares antigens with the genus *Ehrlichia*, particularly with granulocytic species (Jongejan *et al.*, 1989). It is known to produce a serious disease in cattle associated with the distribution of *Amblyomma* spp. ticks in most of sub-Saharan Africa and the Caribbean islands. *Ehrlichia (Cytoecetes) ondiri* is another relative of *E. phagocytophila* enzootic in the highlands and uplands of Kenya (Haig and Danskin, 1962; Davies, 1993).

Fig 2.3 Unengorged male (right) and female (left) of the tick species *Ixodes ricinus* (x5)

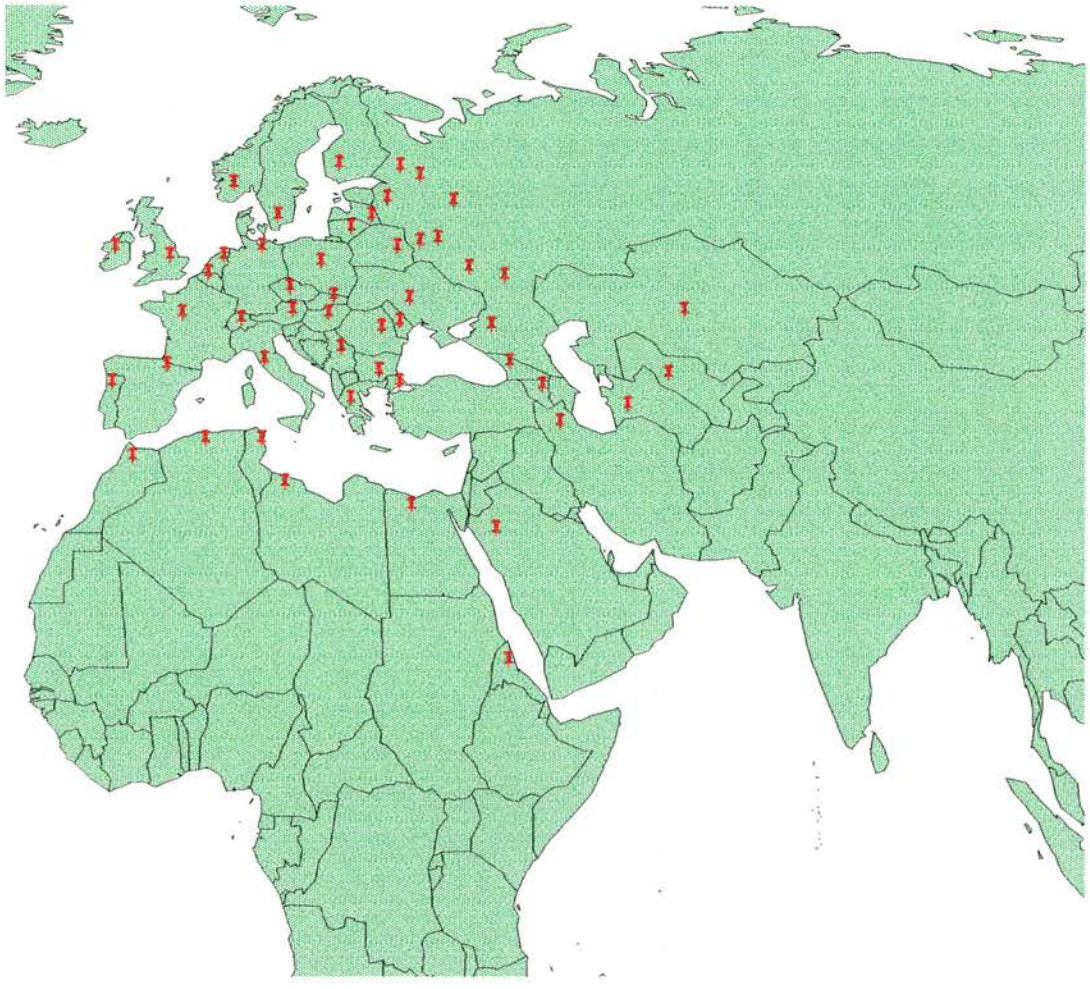


Fig 2.4 Distribution of *Ixodes ricinus* in Eurasia and northern Africa. Adapted from Anderson (1989)

2.2.2 Host range and reservoirs of infection

Ehrlichia phagocytophila appears to have a wide range of susceptible vertebrate hosts which is consistent with the broad range of hosts for its vector, ticks of the genus *Ixodes* (Fig 2.5). Sheep (MacLeod, 1936), cattle (Foggie and Allison, 1960), white-tailed deer (*Odocoileus virginianus*) (Dawson *et al.,* 1994), roe deer (McDiarmid, 1965), dogs (Lewis *et al.,* 1975; Madewell and Gribble, 1982; Greig *et al.,* 1996; Clark *et al.,* 1996), horses (Korbutiak and Schneiders, 1994; Johansson *et al.,* 1995), and humans (Bakken *et al.,* 1994; Chen *et al.,* 1994) are known hosts for granulocytic *Ehrlichia* species. Recently, llamas (Barlough *et al.,* 1997) and jackals (Waner *et al.,* 1999) have been identified as hosts for *E. phagocytophila* infection in US and Israel respectively. Sheep and cattle appear to be the main hosts for *E. phagocytophila* (Fig 2.6) but goats are also susceptible by exposure to ticks or inoculation of infected blood (Macleod and Gordon, 1933; Foggie, 1951).

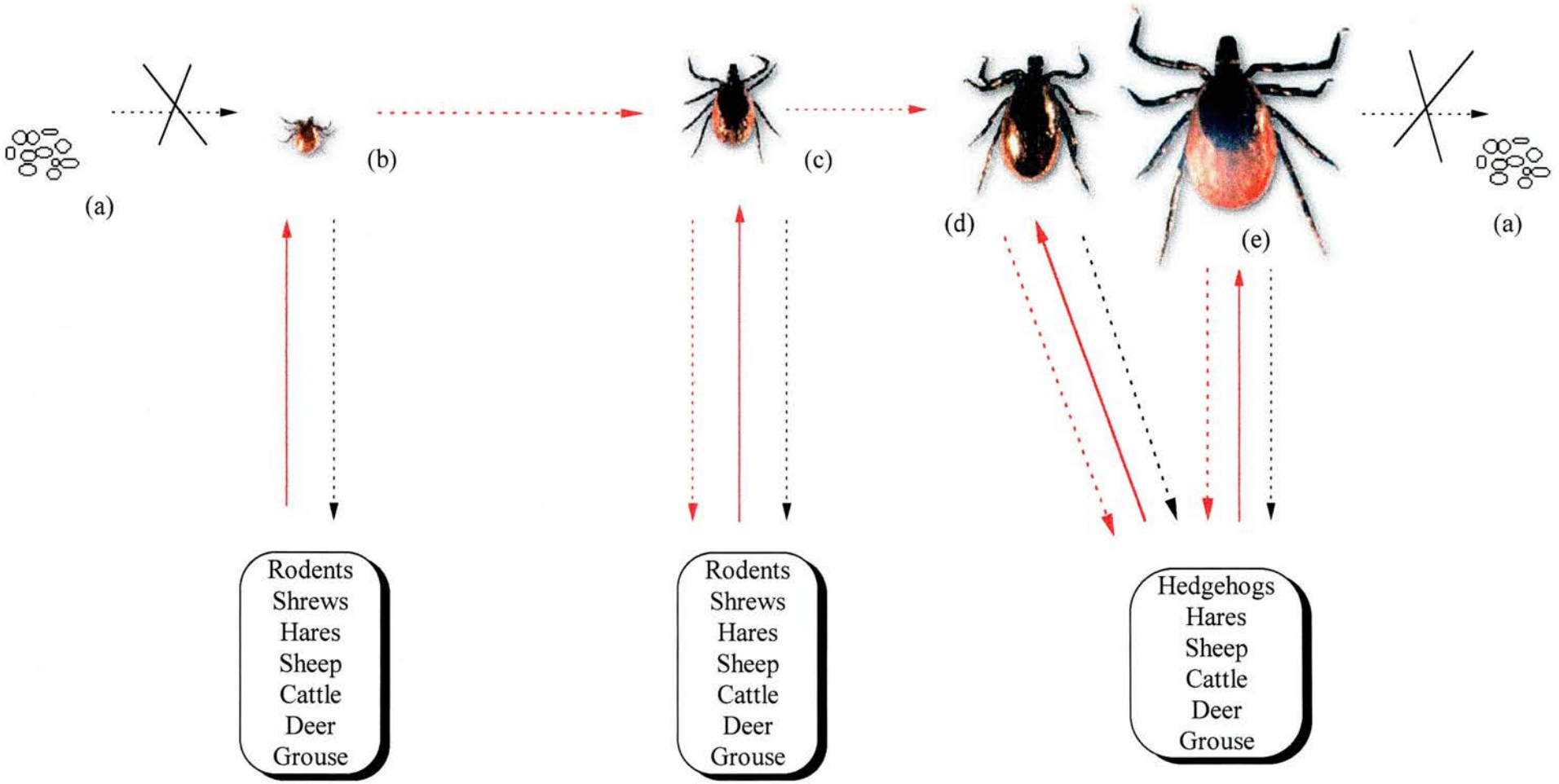


Fig 2.5 Transmission of *Ehrlichia phagocytophila* in *Ixodes ricinus* and main maintenance hosts for the different stages of tick. Only transstadial transmission occurs. Despite female adults acquiring infection from competent reservoirs they do not transmit the bacteria to their progeny. Red arrows indicate transmission of pathogen from ticks to the vertebrate host or vice versa. Black arrows indicate tick feeding. (a) batch of eggs; (b) larvae; (c) nymph; (d) male adult; (e) female adult

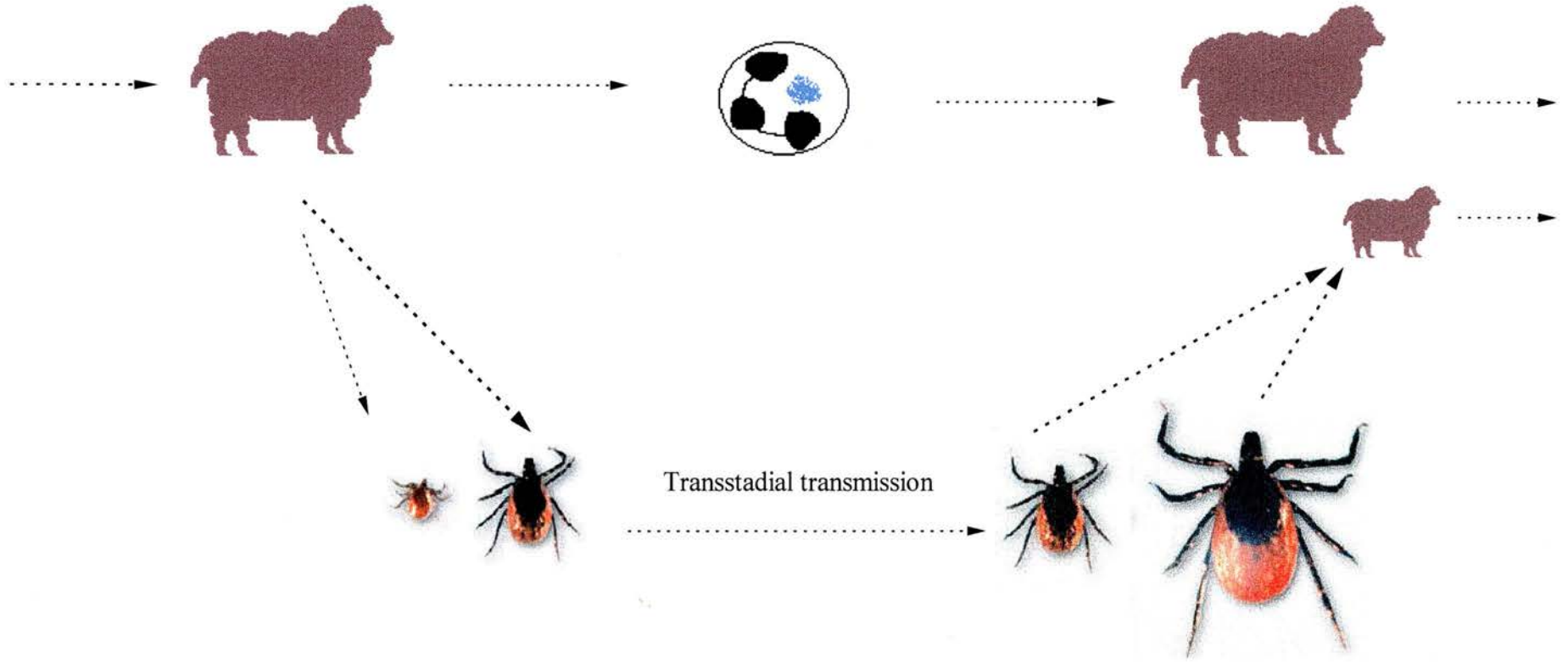


Fig 2.6 Role of carrier sheep in the maintenance of Tick-borne fever. Immature stages of *I. ricinus*, larvae and nymphs, acquire infection when feeding upon reservoir sheep that carry the pathogen within neutrophils. Ticks can transmit the infection transstadially to nymphs or adults. Infected ticks then transmit the pathogen to susceptible lambs

Ehrlichia species were originally believed to be host specific. Host specificity was based on clinical, morphological, and serological observations. However, the recent isolation of DNA from several *Ehrlichia* species, including *E. chaffeensis* agent of monocytic ehrlichiosis in humans, naturally coinfecting dogs (Breitschwerdt *et al.*, 1998) suggests that several *Ehrlichia* species can be transmitted to a variety of hosts in nature. *Ehrlichia equi* can also naturally and experimentally infect dogs (Lewis *et al.*, 1975, Madewell and Gribble, 1982; Madigan and Gribble, 1987). *Ehrlichia risticii*, which was previously regarded as a pathogen specific for horses, has been identified in dogs (Kakoma *et al.* 1994). Moreover, *E. ewingii*, causative agent of granulocytic ehrlichiosis in dogs, has been recently observed infecting humans (Buller *et al.*, 1999).

Many vertebrate hosts seem susceptible to *E. phagocytophila* infection. However, reservoir competence is only demonstrated when a competent vector feeding upon field derived hosts becomes infected and maintains the infection transstadially thus being able to transmit the infection to another vertebrate. Host competence of domestic sheep has been observed (MacLeod and Gordon, 1933). The role of other domestic and wild ruminants as natural competent reservoirs of infection with *E. phagocytophila* still remains to be elucidated by use of transmission experiments. Little is known about how efficiently ticks acquire and maintain infection in sheep populations or if carrier sheep are in fact responsible for the maintenance of infection in the flock. Although sheep appear to be the main reservoir for *E. phagocytophila*, they are known to become resistant to tick infestation soon after the first feeding (Abdul-Amir and Gray, 1987). They develop acquired immunity that impairs full engorgement and reproductive success of ticks on previously exposed hosts. In addition, it is common practice to treat sheep with acaricides, mainly to control *Psoroptes ovis* mites, thus making it difficult for the ticks and *E. phagocytophila* infection to become established within the herd. Early studies assumed that almost all, if not all, ticks and most of the lambs in tick-infested pastures where infected with *E. phagocytophila* (MacLeod and Gordon, 1936, McEwen, 1947). The wording of these reports does not make clear whether it was considered that most populations of ticks were infected or they referred to most

individuals. Recent research has shown contradictory infection prevalence estimates in field collected nymph and adult ticks ranging from very high (49%) to very low (1-2%) (Webster and Mitchell, 1989; Alberdi *et al.*, 1998). However, the studies were carried out in widespread and varied locations and using different techniques thus allowing for the differences. A wide variation of infection with *Cowdria ruminantium* in *Amblyomma hebraeum* ticks from Zimbabwe has been also found (Norval *et al.*, 1990). Thus different species and strains of rickettsial pathogens may have diverse abilities to keep both vertebrate and invertebrate hosts infected which probably depend on many variables including changes in the environmental conditions. It is believed that unfed infected ticks may be an important, if not the main, reservoir of *Cowdria* infection because of their longevity (Camus and Barré, 1987).

The role played by horses and dogs or even cats in the epidemiology of granulocytic *Ehrlichia* is unknown. Blood from HGE patients injected into horses made them develop signs identical to HGE infection in humans. The infection was transmissible to other horses and it induced protection against *E. equi* challenge (Madigan *et al.*, 1996) suggesting they are a single species or at least closely related. Samples from dogs naturally infected with *E. equi* were able to transmit the infection to horses (Madewell and Gribble, 1982). Blood from infected horses when injected experimentally into dogs also induced disease (Walker and Dumler, 1996). All this evidence suggests that horses and dogs could be efficient hosts for *E. phagocytophila* and closely related granulocytic *Ehrlichia*. However, there appears to be poorer transmission of *E. equi* to ruminants and non-human primates (Lewis *et al.*, 1975) and of *E. phagocytophila* to non-ruminants indicating the latter two bacterial strains or species have unique biological properties.

The potential role of white-tailed deer as reservoir of monocytic *Ehrlichia* species has been investigated (Dawson *et al.*, 1994) together with other tick-borne pathogens such as *Borrelia burgdorferi*. A high proportion of white-tailed deer appear to harbour HGE as detected after PCR amplification suggesting they are persistently infected and could provide a high rate of transmission to *I. dammini* ticks (Dawson *et al.*, 1996; Walker and Dumler, 1996; Walker *et al.*, 1996). Roe deer are

hosts for *E. phagocytophila* in Europe (McDiarmid, 1965). Early attempts using blood from roe deer were not infective for sheep or goats. In addition, blood smear examination did not show any evidence of *E. phagocytophila* infection (Foggie and Allison, 1960). However, blood from only three animals was collected and during the winter when there is lower tick activity and bacterial circulation. Neither white-tailed deer nor roe deer appear to be competent reservoirs for *Borrelia* spirochetes (Telford *et al.*, 1988; Jaenson and Talleklint, 1992). They have low susceptibility to *Borrelia* infection thus their spirochaetaemia does not reach the necessary threshold to infect ticks. However, white-tailed deer are believed to be the most important factor for the spread and intensification of *I. dammini* populations acting as tick amplifier hosts (Spielman *et al.*, 1985).

Wild rodents are also regarded as potential reservoirs since they are able to harbour a wide variety of pathogens such as *Borrelia burgdorferi*, *Babesia microti*, *E. chaffeensis* and *E. equi* (Magnarelli *et al.*, 1997; Nicholson *et al.*, 1998). They can act as intermediate hosts for *Ixodes* ticks and appear to be involved in the transmission of granulocytic ehrlichiosis in North America. Recent studies (Telford *et al.*, 1996) confirmed the transmissibility of a human derived strain of HGE (NCH-1) by serial blood passages in mice. Field white-footed mice (*Peromyscus leucopus*) were able to infect laboratory reared ticks indicating that they are reservoirs for HGE in addition to Lyme disease spirochetes and human babesiosis caused by *Babesia microti* (Telford *et al.*, 1996, Walls *et al.*, 1997; Nicholson *et al.*, 1998).

Horses and dogs in Europe appear to be infected with the same agent as showed by Johansson *et al.* (1995) but it is unlikely that human cases of disease are associated with pet ownership or contact with animals as for human monocytic ehrlichiosis (Eng *et al.*, 1990). The identification of a competent reservoir of infection for the granulocytic species appears cumbersome. Humans, horses and dogs are probably dead-end hosts for granulocytic *Ehrlichia*. It is unlikely that humans are a competent reservoir or amplifier host for any disease transmitted by *Ixodes* because these ticks do not feed well enough on humans to maintain a transmission cycle. Horses harbour *E. equi* only during the acute phase of the disease and they are not effective reservoirs for ticks (Lewis, 1976). The duration of bacteremia in human

cases is not known, the currently available methods are not able to detect the pathogen beyond the acute phase and it is therefore assumed to be limited. However, Telford *et al.* (1996) demonstrated the persistence of the bacteria after splenectomy of mice inoculated with a HGE strain. Murid and cricetid rodents appeared to sustain chronic infection by the agent of HGE as demonstrated by reproduction of the infection after subinoculation of blood from mice when no parasites were observed in blood smears even after splenectomy (Telford *et al.*, 1996). *Peromyscus* and *Neotoma* species of rodents samples have shown seroreactivity to HGE by IFA with a prevalence as high as 23% in some areas of the United States where ticks were also common (Nicholson *et al.*, 1998).

The presence of numerous seroreactive species suggests that multiple enzootic cycles for maintaining granulocytic *Ehrlichia* exist and that alternative vertebrate hosts and perhaps vectors remain to be identified.

2.2.3 Vector ecology and transmission

Ixodes species associated with granulocytic *Ehrlichia* are three host ticks (Fig 2.4). Larvae, nymphs and adults (males and females) feed on three different vertebrate hosts. Larvae feed on a wide variety of hosts including rodents, hares, sheep, cattle, deer, and grouse. After the blood meal which last for 3-14 days, they detach from the host into the herbage litter layer where they moult to nymphs. Nymphs ascend the vegetation and wait to attach and feed on the same hosts as larvae then moult to adults. Female ticks have their meal on medium to large mammals (hares, hedgehogs, sheep, and deer), lay a batch containing from 500 to 3000 eggs then die. Male ticks usually do not feed, they can mate off the host but they may be found on the host with the females (Fig 2.5). The whole cycle takes from 2 to 3 years in the UK depending on the environmental conditions (Sonenshine, 1993). It is believed that *E. phagocytophila* infection is reintroduced at each season of tick activity persisting in sheep stock grazing in tick-infested pastures throughout life. Foggie (1951) demonstrated positive reactions in sheep at all times of the year by taking random blood samples from animals on tick-infested farms. Larvae can pick up the infection when feeding on an infected host, and maintain the infection

transstadially to the nymph and adult stages (MacLeod and Gordon, 1933). The disease is seasonal and associated with the activity of the ticks during the springtime. Outbreaks in autumn have been recorded in some areas due to a second period of activity of a separate population of ticks.

Tick transmission has been demonstrated for *E. phagocytophila* (MacLeod and Gordon, 1933; MacLeod, 1936) in sheep, although occasional mechanical or other arthropod vectors like the sheep ked were not ruled out. Only females and nymph ticks were considered able to transmit the disease since transovarial transmission has been demonstrated not to occur (MacLeod and Gordon, 1933; MacLeod, 1936) or at a very low efficiency. *Ixodes* ticks are also vectors for *E. equi* in horses (Richter *et al.*, 1996). DNA from the HGE has been also amplified by PCR in *Ixodes* ticks (Magnarelli *et al.*, 1995b; Pancholi *et al.*, 1995; Reed *et al.*, 1995; Des Vignes and Fish, 1997). The seasonal patterns of human granulocytic ehrlichiosis, the frequent association of outdoors activity, rural environments and/or tick exposure prior the development of symptoms all suggest *Ixodes* ticks as vectors of HGE causing human disease (Pancholi *et al.*, 1995). *Ixodes scapularis* (*I. dammini*), the black-legged (or deer) tick appears to be the main vector for the *E. equi*-like agent of HGE in the United States in addition of *Borrelia burgdorferi* and *Babesia microti* (Magnarelli *et al.*, 1995a; Pancholi *et al.*, 1995). Telford *et al.* (1996) showed vector competence of *I. dammini* ticks for a HGE strain adapted to mice. *Ixodes dammini* larvae feeding on those hosts acquired infection and remained infective when moulting to nymphs, and thus were able to act as competent vectors. When experimentally infected nymphs were used to infect mice all of the latter became infected showing a highly efficient transmission. The pathogen was observed in blood smears for 10 days but persisted for as long as 6 weeks in immunocompromised (B cell deficient) mice. There is also evidence that implicates *Dermacentor variabilis* (American dog tick) as vectors for HGE (Bakken *et al.*, 1994; Magnarelli *et al.*, 1995b; Pancholi *et al.*, 1995; Richter *et al.*, 1996; Telford *et al.*, 1996). *Rickettsia*-like organisms have been detected in the haemocytes of 6.9% *Dermacentor variabilis* ticks (Magnarelli *et al.*, 1991). Other invertebrates may act as vectors of infection as for *E. chaffeensis* where aberrant cases out of the range of

Amblyomma americanum ticks appear to occur (Maeda *et al.*, 1987; Dumler *et al.*, 1995; Walker *et al.*, 1996). The distribution of granulocytic ehrlichiosis could be wider than believed if some other invertebrates are found acting as biological or mechanical vectors.

2.2.4 Co-infection and co-feeding

Salivary glands of *I. ricinus* ticks are activated by the blood first ingested. During tick attachment, feeding and salivation alternate. There is an increase in the rate of ingestion towards the end of the feeding period, engorgement with blood occurring during the last days of attachment (MacLeod, 1936). The broad host range of the genus *Ixodes* allow them to acquire multiple pathogens (Greig *et al.*, 1996). However, Spielman *et al.* (1984) claimed that one vector cannot efficiently maintain horizontal transmission of pathogens having diverse reservoirs hosts. Piesman *et al.* (1987) showed experimental simultaneous transmission of *Borrelia burgdorferi* and *Babesia microti* via *Ixodes dammini* ticks. Magnarelli *et al.* (1991) detected co-existence of *Borrelia burgdorferi* and rickettsia-like organisms in *I. dammini* midgut and haemocytes respectively. Telford *et al.* (1996) observed that 10% of field collected *I. dammini* (*I. scapularis*) ticks were infected with HGE, 20% of which also contained *Borrelia* spirochetes. Piesman *et al.* (1986) observed 18.6% *I. dammini* nymphs concurrently infected with *Borrelia burgdorferi* and *Babesia microti.* Simultaneous transmission of both pathogens appears to occur in hamsters (Piesman *et al.*, 1987) which suggests a common reservoir host for both organisms and indicates that infection in ticks with one agent does not preclude transmission of other pathogen. In Switzerland, human exposure and co-infection with HGE and *Borrelia burgdorferi* and HGE and tick-borne encephalitis virus has been found (Pusterla *et al.*, 1998b). A risk for coinfection in humans with *E. chaffeensis* and HGE appears to exist in the US in areas where both *Amblyomma* and *Ixodes* ticks are abundant (Wong *et al.*, 1997).

Lyme borreliosis and louping-ill endemic areas may act as indicators of risk for granulocytic *Ehrlichia*. Concurrent infection with *Ehrlichia* and louping-ill virus occurs in field collected individual *Ixodes ricinus* ticks (MacLeod and Gordon,

1932). Field collected *Ixodes dammini* ticks by Pancholi *et al.* (1995) showed almost the same infection prevalence for *B. burgdorferi* and granulocytic *Ehrlichia*. Cattle in the UK present similar seroconversion rates to both *B. burgdorferi* and *E. phagocytophila* (Cranwell and Cutler, 1996) suggesting a common risk of infection with either agent in Lyme disease endemic areas.

Tick-transmitted protozoa have been reported to occur in British sheep: *Babesia capreoli* probably derived from red deer (Reed *et al.*, 1976), *Babesia motasi* isolated from *Haemaphysalis punctata* ticks (Lewis and Herbert, 1980) and *Theileria ovis* isolated from sheep and also associated with *H. punctata* ticks (Lewis *et al.*, 1981). None of them are believed to be particularly pathogenic unless co-infecting the same animal.

In louping-ill virus transmission cycles non-viraemic and even immunised hosts can amplify the infection when supporting infected larvae and nymphs that transmit the infection to closely located co-feeding ticks through non-systemic transfer at the feeding sites (Hudson *et al.*, 1995). In this context co-feeding refers to the transmission of pathogens from infected to non-infected ticks when they feed closely at the same site on a vertebrate host without systemic viraemia. Thus herd immunity through vaccination does not avoid the persistence of louping-ill virus in tick populations. Co-feeding transmission also occurs for other *Ixodes*-borne infections like tick-borne encephalitis virus (Labuda *et al.*, 1993) and *Borrelia burgdorferi* (Gern and Rais, 1996). The contribution of non-systemic transmission to pathogen amplification varies. For instance, tick-borne encephalitis virus attains greater amplification via co-feeding when compared to systemic transmission but the opposite occurs for Lyme borreliosis (Randolph *et al.*, 1996). This appears to be associated with the duration of infectivity of the reservoir vertebrate host, which is shorter in the case of tick-borne encephalitis virus. Nothing is known about the role of co-feeding in the transmission of granulocytic *Ehrlichia* in refractory hosts that support immature ticks.

2.2.5 Susceptibility and innate resistance

Young lambs are more resistant to TBF infection, though they undergo febrile symptoms and parasitaemia, their clinical signs are inapparent (Stuen, 1993). However, some authors have claimed that very young lambs (less than 2 weeks-old suckling lambs) show most overt cases of the disease (Scott, 1984). It appears that neutrophil numbers in lambs decrease from the second week of life (Jain, 1993) which can account for the difference in susceptibility with increasing age of lambs. After patent parasitaemia sheep can become carriers and be responsible for the maintenance of infection in the flock. Every year young lambs join the flock and they are fully susceptible to successful tick feeding and *Ehrlichia* infection. If there is colostral maternal immunity it does not appear sufficient to inhibit the infection in lambs although it makes the disease milder.

Foggie (1951) observed that calves were resistant to ovine strains of TBF although their blood was infective for sheep. Older cattle showed fever and parasitaemia when inoculated with the same strains. Guinea pigs, rabbits and mice appeared resistant to the disease and their blood was not infective. However, recent experiments with a human derived granulocytic *Ehrlichia* indicated that it was transmissible to mice (Telford *et al.*, 1996).

Young horses also appear to show less severe signs after *E. equi* infection, very young foals (2 days- 9 months) only had fever that was accompanied by slight depression and oedema in older horses (1-2 years) (Gribble, 1969). Foals born from an immune mare were more resistant to clinical signs of the disease after inoculation with *E. equi*, although they showed characteristic morulae within granulocytes and fever.

Little is known about the innate resistance of young animals from other species. To extrapolate from the situation in sheep, wild populations of deer could sustain infection with granulocytic *Ehrlichia* when very young fawns become exposed to infected ticks when still resistant to the disease. Then they may also become carriers and act as competent reservoirs for the pathogen in the wild.

Innate resistance in young animals to other tick-borne rickettsial infections such as heartwater (Neitz and Alexander, 1941) or anaplasmosis (Corrier and Guzmán, 1977) also appears to occur. Resistance to *Cowdria ruminantium* in younger animals was suggested to be independent of the immune status of the dam (Neitz and Alexander, 1941) and of the breed (DuPlessis and Malan, 1988).

2.2.6 Zoonotic importance

Early research showed that non-human primates were susceptible to *E. equi* experimental infection (Lewis *et al.*, 1975) suggesting the bacteria were potentially zoonotic. The induced disease was characterised by mild fever and anemia that resolved within a week. Humans recently appeared to be infected with granulocytic *Ehrlichia* in Europe and the United States (Chen *et al.*, 1994, Dumler and Bakken, 1995; Petrovec *et al.*, 1999). It is not clear if the rising human cases occur with species closely related but different from *E. phagocytophila* and *E. equi.* It is relevant to determine the conspecificity of granulocytic *Ehrlichia* because of the zoonotic implications and epidemiology of infection in humans. The disease is more frequent in middle-aged males with frequent outdoors activities. However it can also affect children and elderly people in which the immunosuppression associated with the infection poses a special risk (Foggie, 1956).

Inoculation of HGE strains into horses is known to induce a disease similar to equine ehrlichiosis associated with *E. equi* (Dumler *et al.*, 1995; Madigan *et al.*, 1995; Madigan *et al.*, 1996; Chang *et al.*, 1998). This indicates they are highly related species and horses, and perhaps humans, are susceptible to both.

There is serological evidence of co-infection with *Babesia microti*, *Borrelia burgdorferi* and the agent of HGE in the same human patient (Mitchell *et al.*, 1996). However, confirmation is needed by means of cultivation or PCR methods since cross-reactions between heat shock proteins of bacterial species are likely to occur (Scorpio *et al.*, 1994). Experimental transmission of *Borrelia* and *Babesia* has been attained by individual *Ixodes* ticks (Piesman *et al.*, 1987) thus emphasising the potential risks after a single tick bite in humans and animals.

2.3 Pathogenesis

2.3.1 Incubation period

The incubation period after infection with *E. phagocytophila* ranges from 4 to 13 days depending on the type of infection (tick exposure or subinoculation of infected blood). Reinfected animals show longer incubation periods indicating an anamnestic antibody-dependent response (Woldehiwet and Scott, 1982a).

2.3.2 Clinical signs

Ehrlichia phagocytophila is characterised by producing a mild disease in sheep although it can induce abortion in primary infected pregnant ewes. Experimentally, 3-4 days after the pathogen is inoculated into the host it develops a transient high fever (40.5-42°C) that lasts for 4-5 days. The fever rises before any other clinical sign is observed. Blood is infective for sheep 24 h after experimental inoculation (Snodgrass, 1974). In blood smears the neutrophils show characteristic inclusion bodies (morulae) in the cytoplasm when stained by Giemsa. Clinical signs range from inapparent to mild or severe depending on the age and status of the animal, but they are usually depressed and reluctant to move. The patent period lasts for 5-6 days during which the number of neutrophils is increased followed by a severe lymphopenia and neutropenia. The associated neutropenia often activates latent infections or exacerbates the symptoms in cases of purulent pneumonia associated with *Pasteurella* (Foggie, 1951), *Staphylococcus* septicaemia inducing arthritis in lambs (tick pyemia) (Taylor *et al.*, 1941; Brodie *et al.*, 1986), louping-ill (MacLeod and Gordon, 1932), or listerial septicaemia (Gronstol and Ulvund, 1977) due to the immunosuppression associated with *Ehrlichia*. Concurrent infections induce more severe conditions and increase the mortality rate as demonstrated with *Chlamydia psitacci* (Munro *et al.*, 1982) or parainfluenza-3 virus (Batungbacal and Scott, 1982b). Mortality rates are considered to be low (Gordon *et al.*, 1932) although it varies according to different studies from 13% (Foggie, 1951) to 24% (Jamieson, 1947). Deaths are usually associated with the introduction of naive animals to tick infested pasture or exacerbation of concurrent disease.

Corticosteroid injection appears to increase the absolute number of neutrophils, including the infected ones, but this effect does not persist (Woldehiwet and Scott, 1982a). Occasional relapses may occur, the animals show parasitaemia for 1 or 2 days, and sometimes fever, 3-4 weeks after primary infection or even later (Stuen *et al.*, 1998).

Most animals are believed to become carriers after patent parasitaemia as demonstrated by subinoculation of blood into susceptible animals (Scott, 1984; Stuen, 1993). Carrier state seems to last for months or even years in sheep (MacLeod, 1936). The presence of carriers may be responsible for the maintenance of infection in the population until the next generation of fully susceptible animals arrives.

Tick-borne fever infection induces dramatic changes in the relative proportions of leukocytes. Initially, neutrophilia and lymphopenia occur then a pronounced neutropenia and thrombocytopenia follows. Neutropenia lasts for 1-3 weeks and during that period *E. phagocytophila* is not found in blood smears probably because of a very low level of infection. Neutropenia is believed to be associated with the accumulation of neutrophils in marginal pools, perhaps spleen, and the sequestration and destruction of infected cells. The animals normally recover with or without antibiotic treatment. Lymphocytopenia is related to the reduction of peripheral B-lymphocytes (Batungbacal *et al.*, 1982) inducing suppression of the antibody response to other antigens (Batungbacal and Scott, 1982a). Research has identified a platelet migration inhibition factor (PMIF) produced by lymphocytes in dogs with acute *E. canis* infection (Kakoma *et al.*, 1978). Thrombocytopenia is a common feature in granulocytic *Ehrlichia* infection (Foster and Cameron, 1968; Bakken *et al.*, 1996), which could be associated with a similar factor.

The disease is inapparent in endemic situations where the animals are constantly challenged by infected ticks. However when livestock are moved from tick free areas to tick-infested pastures an abortion storm and some mortality are expected to occur (Stamp and Watt, 1950; Wilson *et al.*, 1964; Jones and Davies, 1995). The severity of the disease also depends on the virulence of the strain. After a sudden rise in temperature, fever can rapidly drop or fluctuate for 10-12 days gradually declining, then the bacteria disappears and neutrophil counts return to normal.

Recent natural and experimental infections in dogs with a Swedish isolate genetically identical to the HGE agent at 16S rDNA level (Egenvall *et al.*, 1997, 1998) induced clinical signs and histopathological findings in agreement with the classical picture of TBF. Those symptoms included fever, leucopenia, thrombocytopenia, reluctance to move, and lack of appetite. The dogs also showed the characteristic morulae inside the neutrophils and a rise in antibodies to *E. equi* which persisted for several months after infection (Egenvall *et al.*, 1997). Granulocytic *Ehrlichia* infection in horses induced limb oedema (Johansson *et al.*, 1995) which is a typical sign of the disease induced by *E. equi* (Gribble, 1969).

Human granulocytic ehrlichiosis is a moderate to severe disease. Clinical signs are non-specific influenza-like symptoms with fever, severe myalgia, chills and headaches (Bakken *et al.*, 1994). The incubation period ranges from 1 to 60 days with a median of 8 days (Bakken *et al.*, 1996). Haematological changes also occur including leucopenia and thrombocytopenia (Chen *et al.*, 1994). In addition, alkaline phosphatase, lactate dehydrogenase and aminotransferases appear elevated (Bakken *et al.*, 1994; 1996) suggestive of hepatic injury. The extent of morbidity and mortality from HGE infection is not yet well defined. Fatal cases of HGE have been associated with secondary opportunistic fungal or viral infections (Bakken *et al.*, 1994; Chen *et al.*, 1994; Walker and Dumler, 1996) suggesting that immunosuppression occurs as for sheep infected with *E. phagocytophila* (Foggie, 1956). Thrombocytopenia and coagulopathy have been observed in severe infections predisposing to haemorrhagic complications. Neutropenia and other host defence defects may predispose to opportunistic infections. Furthermore, deaths have been associated with severe T-cell dysfunction (Bakken *et al.*, 1994). However, most of the infections are self limiting if uncomplicated (Bakken *et al.*, 1996; Dumler *et al.*, 1997; Petrovec *et al.*, 1997).

HGE and *E. equi* infections induce similar diseases in horses (Madigan *et al.*, 1995). Furthermore, horses inoculated with HGE are resistant to the challenge with *E. equi* (Barlough *et al.*, 1995). Clinical signs include non-specific influenza-like symptoms and abnormal haematological profiles similar to the changes induced in sheep after *E. phagocytophila* infection: leucopenia, thrombocytopenia, anemia and characteristic morulae inside granulocytes (Chen *et al.*, 1994; Dumler and Bakken,

1996; Greig *et al.*, 1996; Walker and Dumler, 1996). In addition, horses present oedema usually restricted to the legs, central nervous system depression and ataxia (Stannard *et al.*, 1969; Gribble, 1969). Lymphocytosis is also common 7-10 days after the clinical disease (Stannard *et al.*, 1969).

2.3.3 Pathological findings

Gross changes are usually absent apart from spleen enlargement, petechiation of thymus and intestinal mucosa, colon, serosal and subendocardial haemorrhages and hydropericarditis in lambs (Gordon *et al.*, 1932; McEwen, 1947; Campbell *et al.*, 1994). Microscopical examination of liver and spleen reveals the presence of *E. phagocytophila* in Kupffer cells and neutrophils. Large mononuclear cells can be observed in alveolar septa and the pathogen inside alveolar macrophages (Munro *et al.*, 1982). Campbell *et al.* (1994) described mild histopathological changes that increased in severity according to the duration of the infection. Those included pulmonary alveolitis, lymphoreticular reactions in lymph nodes and spleen, mesangial glomerulitis and choroiditis in the cranial nervous system. In tissues, *Ehrlichia* was associated with lymphoid and macrophagic cells but it was difficult to find in granulocytes. There appeared to be a generalised pathological response centred on mononuclear antigen processing cells. A pathognomonic feature was also described, two types of cytoplasmic inclusions were observed, representing stages of evolution of the agent in tissue cells similar to those described by Woldehiwet and Scott (1982c) in granulocytes.

Pathological changes in cattle after infection with *E. phagocytophila* are similar to those induced by *Cytoecetes ondiri*, including multiple petechial haemorrhages (Foggie and Allison, 1960). Hudson (1950) also mentioned mild cellular changes and loss of lymphoid tissue.

Gross changes induced by *E. equi* infection are mild or absent. Pathological changes in horses include oedema, petechiae, echymoses in subcutaneous tissues and fascia of the legs with vasculitis (Stannard *et al.*, 1969; Gribble, 1969). Mild inflammatory lesions were also found in kidneys, heart, brain and lungs. Similar pathology is observed after experimental inoculation of horses with HGE agent

including follicular hyperplasia of lymphoid tissues. DNA from HGE experimental infection in horses can be detected after PCR amplification in muscle, fascia, joints, peritoneum and adrenal glands of horses well after the organism was undetectable in blood smears (Chang *et al.*, 1998). It is believed that granulocytic *Ehrlichia* persists in poorly vascularised connective tissues where antibodies or antimicrobial agents have difficulty to reach thus evading host immune responses.

Changes at necropsy in dogs experimentally inoculated with granulocytic *Ehrlichia* are similar to those mentioned above. Spleens appear enlarged and congested, microscopically showing reactive hyperplasia with increased numbers of macrophages and plasma cells in the red pulp (Egenvall *et al.*, 1998). Livers also showed enlargement and non-specific reactive hepatitis.

Human patients who died after granulocytic infection presented pulmonary infiltrates, yeast pneumonitis (*Candida* and *Cryptococcus* species), and herpes simplex virus type I oesophagitis (Bakken *et al.*, 1994).

2.4 Immunity

Infection with *E. phagocytophila* induces initial production of IgM followed by IgG immunoglobulins. Immunity appears to be sterile in cattle and for a very short period (Hudson, 1950; Foggie and Allison, 1960). Antibody response lasts longer in sheep and the removal of the spleen frequently induces the reappearance of the bacteria in the blood stream (Foggie, 1956), which confirms the carrier state in sheep.

2.4.1 Humoral response

High antibody titres of IgM and IgG are detectable one week after the infection. They reach the peak at 3-4 weeks and may persist for as long as two years in sheep. After recovery, the animals are usually protected against the homologous challenge but the response to the heterologous challenge is variable.

High levels of IgM are detected for long periods after recovery in sheep (Woldehiwet and Scott, 1982a) indicating continuous exposure of the immune system with the pathogen and suggesting carrier state. Paxton and Scott (1989)

observed specific anti-*E. phagocytophila* IgM (by counter-immunoelectrophoresis) and IgG (by IFAT) antibodies on the fifth day after the appearance of parasitaemia. The levels of antibodies remained high for at least 8 weeks and persisted for 6 months.

Ehrlichia phagocytophila infection appears to impair primary and secondary antibody responses to some antigens such as tetanus toxoid and influenza virus. However, higher antibody titres to *Actinomyces pyogenes* have been found in experimentally inoculated sheep when compared to uninfected controls (Larsen *et al.*, 1994). This is believed to be associated with an increased exposure to *A. pyogenes* antigen, because of the neutropenia induced by *Ehrlichia* infection, and therefore enhanced antibody response to the bacteria, which circulates freely in the blood stream.

Immunosuppression lasts for at least six weeks after experimental inoculation with *E. phagocytophila*, which can interfere with vaccination responses as shown by Batungbacal and Scott (1982a) against blackleg vaccine. Hypoimmune responses have been also described in mice infected with *E. risticii* (Rikihisa *et al.*, 1987).

2.4.2 Cellular response

Little is known about the cell-mediated immunity in granulocytic *Ehrlichia* infections. Soluble factors appear to impair parasitised and normal leukocyte migration in vitro (Foster and Cameron, 1970b; Woldehiwet and Scott, 1982d) and also inhibition of phagocytosis and killing. Early lymphocytopenia is followed by severe neutropenia (Taylor *et al.*, 1941; Scott, 1984) which appears to predispose to other infections or increase the severity of concurrent conditions such as mastitis or latent brucellosis (Juste *et al.*, 1989). In addition, co-infection of *E. phagocytophila* and louping-ill appears to favour virus invasion of the central nervous system (Reid *et al.*, 1995). However, no cell-mediated immunity has been associated to this process.

Lymphocyte proliferation studies (Woldehiwet, 1987; Larsen *et al.*, 1994) indicated lower responses to the lectin mitogens Concanavalin A (ConA), phytohaemaglutinin (PHA) and pokeweed mitogen (PWM) in experimentally

inoculated sheep when compared to the control group. The same result was obtained when mixing serum of infected animals with serum from healthy donors, but the suppression was stronger to ConA than to PHA and PWM, suggesting the involvement of inhibitory serum factors derived perhaps from dead granulocytes.

Severe T cell dysfunction has been associated with fatal cases of human granulocytic ehrlichiosis (Bakken *et al.*, 1994).

2.4.3 Immunogenic and antigenic variation

Immunity to *E. phagocytophila* after recovery appears to be variable. Some authors have claimed a solid immunity to the disease in sheep (Stamp and Watt, 1950) whereas other showed it was short-lived in cattle (Hudson, 1950). Foggie (1951) indicated that immunity depended on premunition whereas Hudson (1950) and Tuomi (1967c) claimed that carrier state was not necessary to maintain immunity to the disease. Immunity does not appear to be absolute, sheep react to challenge but they are protected against serious manifestations of the disease. Resistance to reinfection develops 5 weeks after primary infection and wanes at 5-6 months post infection (Foggie, 1951).

There are immunological differences between strains of *E. phagocytophila*. Most sheep react clinically against heterologous infection but not to the homologous challenge when they are experimentally inoculated at 5 weeks after primary infection, when believed to be resistant (Foggie, 1951). Challenge of experimentally inoculated sheep induces the reappearance of the bacteria within granulocytes and sometimes fever but only in animals with low antibody titres. Secondary infections with the homologous strain are usually milder with longer incubation periods but neutropenia and lymphocytopenia also occur (Woldehiwet and Scott, 1982a). Complement fixation (CF) titres rose significantly one week after challenge in reacting sheep and were also significantly higher than in non-reacting sheep. Antibody responses were faster after challenge indicating an anamnestic response. Non-reacting sheep showed lower increase in their antibody titres by CF indicating a state of premunition.

2.4.4 Persistent infections and carrier state

Persistent infection of animals is an indication of host competence for the maintenance of *E. phagocytophila* infection in nature. Tick or other blood-sucking vectors may acquire the bacteria after feeding on those animals. After infection with *E. phagocytophila* some sheep become carriers. The ability to induce carrier state appears to differ dramatically between strains of *E. phagocytophila* (Scott, 1979). This state persists for only a few weeks, thereafter there is a steady decline and, after a year, very few carriers are detected. It appears that frequent exposure or challenge with the bacteria does not alter the rate of decline in the proportion of sheep that were carriers (Scott, 1977). Carrier animals appeared solidly immune but, more interestingly, 30% of non-carrier sheep were also immune to challenge (Scott, 1977). The existence of sterile immunity in sheep suggests inactivated vaccines are likely to protect the animals against granulocytic *Ehrlichia* spp. infections.

Little is known about the location of *E. phagocytophila* in persistently infected sheep. The pathogen is believed to survive for months in sheep as demonstrated by the detection of the organism in blood smears after splenectomy at 48 weeks (Foggie, 1951) or even two years after primary infection (Stuen, 1993). There is no specific tissue tropism for the bacteria (Campbell *et al.*, 1994) as opposed to other *Ehrlichia* species (Rikihisa, 1991) although it seems to have a preference for reticuloendothelial cells and macrophages of various tissues (Campbell *et al.*, 1994). Macrophages have a longer life span than neutrophils, from weeks to years (Jain, 1993), which can account for the persistence of *E. phagocytophila* within the host. Splenic granulocytes in human patients that died showed the presence of *Ehrlichia* (Chen *et al.*, 1994). Gordon *et al.* (1932) observed the persistence of *E. phagocytophila* in blood, mesenteric glands, spleen and central nervous system. A closely related *Rickettsia*, *Cowdria ruminantium*, shows also a tropism for endothelial cells of the central nervous system (Cowdry, 1925) and can multiply within granulocytes (Logan *et al.*, 1987). *Ehrlichia equi* and granulocytic *Ehrlichia* in horses have also a preference for endothelial cells as demonstrated by the presence of limb oedema in infected animals (Gribble, 1969; Johansson *et al.*, 1995). Blood from experimentally inoculated lambs is still infective after 6 months (Stuen *et al.*, 1998)

and morulae are detectable in blood smears when 400-1000 neutrophils were examined for infection. However, smears from aspirates of sternal bone marrow at week 26 after infection did not show any inclusion or abnormal condition.

Woldehiwet and Scott (1982a) suggested that high titres by complement fixation were linked to carrier state in sheep since only sheep with low titres reacted to experimental challenge with fever and low parasitaemia indicating that antibodies were protective towards reinfection. They also found persistent IgM titres that started to decline at 6 weeks after experimental inoculation indicating a low grade of stimulation by the antigen. However, it has been suggested that immunosuppressive agents such as *E. phagocytophila* may induce prolonged IgM synthesis (Svehag and Mandel, 1964). Continuous production of IgM has also been found after *E. canis* infection in dogs (Weisigar *et al.*, 1975).

It is controversial if *E. equi* can induce persistent infections as appears to occur in several *Ehrlichia* species (Rikihisa, 1991). Blood collected from ponies 3 months after *E. equi* infection induced mild clinical signs when inoculated in susceptible ponies (Nyindo *et al.*, 1978). It has been also suggested that HGE induces persistent infection in humans (Dumler *et al.*, 1995; Magnarelli *et al.*, 1995a) as for *E. chaffeensis* in humans (Dumler *et al.*, 1993), *E. canis* in dogs (Iqbal and Rikihisa, 1994) and *Anaplasma marginale* in livestock (Knowles *et al.*, 1996). Human infection with HGE appears to induce persistence of the pathogen for at least 30 days after the onset of illness (Dumler and Bakken, 1996). Chronic cases of granulocytic ehrlichiosis in dogs have been described (Egenvall *et al.*, 1997) associated with subtle clinical signs and persistent titres to *E. equi*.

2.4.5 Characterisation of ehrlichial antigens and cross-reactivity

HGE appears to have strain pleomorphism in its major antigenic proteins although all antigenic epitopes are cross-reactive (Zhi *et al.*, 1998b). Diversity of major antigens has been also observed among different strains of *E. risticii* and *E. chaffeensis* (Chen *et al.*, 1996). Using recombinant proteins instead of crude antigens avoids cross-reactions with heat-shock proteins and other antigens commonly found in several bacterial species (Kaufmann, 1990; Wong *et al.*, 1997). Granulocytic

Ehrlichia possess a major immunodominant 44-kDa antigen, which is a member of a multigene family recently cloned and characterised by Ijdo *et al.* (1998) and Zhi *et al.* (1998a) for the agent of HGE. A 44-kDa protein was also determined to be characteristic for granulocytic *Ehrlichia* species in immunoblots performed by Dumler *et al.* (1995) using *E. equi* as antigen. No other species of *Ehrlichia* were found to cross-react with the band. The monocytic species *E. chaffeensis* and *E. canis* reacted occasionally but very faintly. An immunoblot profile of 44-kDa plus the presence of a 25, 42 or 100-kDa band was thus considered diagnostic for granulocytic *Ehrlichia* infection. These results are in discrepancy with immunoblots performed by Nyindo *et al.* (1991). In the latter study they found cross-reactions among *E. canis*, *E. sennetsu*, *E. risticii* and *E. equi* due to the presence of shared antigens. Other reports have also found serologic cross-reactions by IFA between *E. phagocytophila* and *C. ruminantium* (Jongejan *et al.*, 1989), *E. chaffeensis* and *E. equi* (Wong *et al.*, 1997), and *E. canis* and *E. sennetsu* (Jongejan *et al.*, 1989). However, they occurred at very low titre indicating non-specific reactions or specific reactions to highly conserved antigens among *Ehrlichia,* and probably other genera of eubacteria (Wong *et al.*, 1997), such as the *groESL* chaperonin homologs (Sumner *et al.*, 1993).

Kolbert *et al.* (1997) created a 45-kDa recombinant fusion protein expressed from a fragment of the *groEL* gene after PCR amplification. It induced a strong antibody response in mice experimentally inoculated with a strain of HGE. No other *Ehrlichia* species were tested. No responses were obtained with antiserum derived from *B. burgdorferi* infected mice or non-inoculated controls indicating there were no cross-reactions to other selected tick-borne pathogens.

2.5 Diagnosis

A tentative diagnosis of TBF can be made based on case history, tick exposure, and clinical signs. Several methods are available to confirm the diagnosis, most of them based on the detection of the pathogen during patent parasitaemia within peripheral blood neutrophils. Subinoculation of blood from the infected

animal and reproduction of the disease in a naive host is another means to confirm the diagnosis.

2.5.1 Differential diagnosis

Tick-borne fever lacks pathognomonic signs, the predominant ones simulate many other febrile conditions of viral or bacterial origin such as pasteurellosis or brucellosis. Foggie and Allison (1960) suggested that many cases were missed in dairy cattle when dry because TBF infection was only presumed after a rapid drop in milk yield that lasted for the whole season. The disease must be suspected when naive lambs show high fever and abortion is seen in pregnant ewes after their movement to tick-infested pastures. The presence of carriers can be demonstrated by subinoculation of blood into susceptible sheep or by detection of high antibody titres directed towards the pathogen.

Clinical signs of granulocytic ehrlichiosis in hosts other than sheep are also non-specific. HGE cases can be misdiagnosed with HME or even other tick-borne diseases such as Lyme borreliosis. Disease in humans caused by granulocytic *Ehrlichia* should be suspected when previously healthy individuals present flu-like symptoms after outdoor activities and/or tick bite. Clinical suspicion should be confirmed with retrospective serology. Low levels of cross-reactivity between *E. chaffeensis* and HGE occur, apparently stimulated by high antibody titres to the granulocytic agent but not the converse (Wong *et al.*, 1997). Some cases of HME outside the geographic range of *Amblyomma americanum* should be reassessed with more specific diagnostic tools. Leukocyte cytoplasmic inclusions have been described in cases of leishmaniosis or Chagas' disease but those illnesses do not occur endemically in temperate zones. Morulae may also be confused with cytoplasmic inclusions such as Döhle bodies and toxic granulations.

2.5.2 Laboratory diagnosis

2.5.2.1 Blood smears

The pathogen is microscopically visible after the staining of blood smears with Romanovsky stains such as Giemsa. It shows a characteristic morulae shape

inside the cytoplasm of the neutrophils. The pathogen is only detectable in blood from approximately days 3 to 10 after infection. Immunocompromised mice can show persistent parasitaemia for as long as 6 months after experimental inoculation with HGE (Telford *et al.*, 1996). Several authors have reported the persistence of the agent in sheep demonstrated by the reappearance of the agent in blood after splenectomy well after the animal were fully recovered (Foggie, 1951). It is suspected that the agent persists at low level undetectable by the available diagnostic tools. Stuen *et al.* (1998) were able to identify *E. phagocytophila* in blood smears from lambs months after the experimental inoculation but only when counting large numbers of neutrophils, from 400 to 1000.

The dramatic haematological changes following infection with *E. phagocytophila* and other granulocytic *Ehrlichia* are characteristic. Peripheral white blood cell and thrombocyte counts should be performed to confirm the diagnosis.

2.5.2.2 Serology

Serological tests are only an indicator of exposure and therefore more useful for epidemiological studies. The use of serological tests for the diagnosis of granulocytic *Ehrlichia* is restricted to the convalescent phase of the disease when antibody responses have been mounted.

Tuomi (1967c) first developed a direct fluorescent test for *E. phagocytophila*. Snodgrass and Ramachandran (1971) then described a complement fixation test (CFT) but they found difficulties in the source of antigen. It was further optimised by Woldehiwet and Scott (1982a) using three different methods to improve the yield of antigen (OS strain). Higher titres were obtained when blood from infected sheep was cultured overnight at 37°C and even higher titres when the blood sample was obtained 6 h after inoculation with betamethasone. Corticoids are known to increase the absolute number of circulating granulocytes (Mishler, 1977; Woldehiwet and Scott, 1982a). Woldehiwet and Scott (1982c) observed that overnight culture of blood increased not only the number of infected cells but also the number of organisms per infected cell.

IgG and IgM immunoglobulin antibodies can be readily detected by IFAT. The method was first developed by Paxton and Scott (1989) using purified infected neutrophils as antigen, then modified by other authors (Hardeng, 1991). It is the test of choice for the serological diagnosis of TBF and it has been used as a surrogate for *E. equi* and HGE diagnosis (Mitchell *et al.*, 1996; Egenvall *et al.*, 1997; Wong *et al.*, 1997). The use of surrogate antigens for serological diagnosis may, however, induce false negative results because the pathogens from different species may not contain all relevant immunodominant antigens. Antibody levels in sheep start rising a week after primary infection and persist high for at least eight weeks. High levels of IgM are detected after recovery in sheep (Scott, 1984) indicating continuous exposure of the immune system to the pathogen. Many drawbacks have been found for IFAT. Visualisation of *E. phagocytophila* is variable due to the existence of different developmental stages (Foggie, 1951) leading to subjective interpretation of the results. Fluorescein labelled Ig attach only to E *phagocytophila* particles and not to the vacuoles containing them (Alani *et al.*, 1987). In addition, it is laborious and requires skilled personnel. IFAT for other agents of granulocytic ehrlichiosis has been described using human promyelocytic leukaemia cells HL60 to grow the bacteria (Goodman *et al.*, 1996, Heimer *et al.*, 1997). However, this is a lengthy procedure and the sensitivity has not been determined yet. Furthermore, cross-reactions with *E. chaffeensis* have been observed (Magnarelli *et al.*, 1995a; Bakken *et al.*, 1996).

Counterimmunoelectrophoresis has been developed for the detection of antibodies to *E. phagocytophila* (Webster and Mitchell, 1988). A peak titre was found at 2 weeks after inoculation, antibodies persisting but slowly declining for 6-10 weeks.

ELISA has been also used for the detection of IgG antibodies to *E. phagocytophila* (Larsen *et al.*, 1994), with a sonicated suspension of infected granulocytes as antigen, processed following Dutta *et al.* (1987) methods for *E. risticii*, causative agent of Potomac Horse Fever. ELISA using Percoll purified antigen has been developed for *Cowdria* (Neitz *et al.*, 1986) but the yield of antigen did not appear to be sufficient.

Long-term cultivation for *E. phagocytophila* has been hitherto unsuccessful due to their specific association with neutrophils that are fragile host cells thus hindering the development of further immunodiagnostic tests and detailed analysis of the biology of the pathogens. Cultivation has been however attained in the case of *E. equi* in IDE8 tick cell line (Munderloh *et al.*, 1996b) and the HGE agent in HL60 cells of the human promyelocytic line (Goodman *et al.*, 1996) and tick cell lines (Munderloh *et al.*, 1999).

2.5.2.3 PCR amplification of ehrlichial DNA

Since the diagnosis of granulocytic ehrlichiosis in hosts other than ruminants or horses and the discovery of their close relationship to *E. phagocytophila*, much interest has grown towards the molecular diagnosis of such pathogens. PCR assays based on 16S rDNA sequence are ideal for the detection of pathogenic bacteria that are difficult to grow and isolate in the laboratory (Anderson *et al.*, 1991; McDade and Anderson, 1996). Primers for the amplification of fragments from the 16S ribosomal RNA gene, known to be very conserved between bacteria and widely used for phylogenetic studies, have been extensively published for rickettsial species (Wilson *et al.*, 1990; Weisburg *et al.*, 1991; Van Vliet *et al.*, 1992; Roux and Raoult, 1995) and specifically for granulocytic *Ehrlichia* (Chen *et al.*, 1994; Johansson *et al.*, 1995; Pancholi *et al.*, 1995; Barlough *et al.*, 1996; Engvall *et al.*, 1996; Massung *et al.*, 1998). Unfortunately, 16S is unable to differentiate between granulocytic species from the same genogroup unless combined with sequencing which is beyond the scope of routine laboratory diagnosis. Most PCR primers for the 16S rRNA gene are therefore designed to amplify DNA for the whole *E. phagocytophila* genogroup.

On the other hand, PCR technique is not more sensitive than the standard staining procedures for the detection of the pathogen in blood smears during patent parasitaemia (Telford *et al.*, 1996, Stuen *et al.*, 1998) and its use as a diagnostic tool is thus restricted to the acute phase of the disease. Despite PCR not being sensitive enough to detect latent infection with granulocytic *Ehrlichia* it can be very useful to confirm the identity of the pathogen. A gene coding for the *groE* operon (heat shock protein) is also well conserved but it shows more variation and therefore it can be

more useful to distinguish between closely related species (Sumner *et al.*, 1997; Petrovec *et al.*, 1999). PCR for this gene in combination with RFLP or specifically designed probes could readily differentiate between closely related bacterial species. However, in most cases it would be enough to determine the granulocytic nature of the pathogen to start a prompt treatment.

2.6 Control and prevention

The control of the disease may be directed towards control of the vector population and good management of the flocks. However, many aspects related to the epidemiology of the disease are still unclear, like the reservoir hosts for the bacteria and the prevalence of infection in ticks.

The emergent tick-borne infections may be associated with changing vector-host ecology and increased clinical knowledge. There is still the need for the development of better laboratory methods and epidemiological surveillance to yield information about the source of infection, geographic distribution, and risk factors for the disease.

2.6.1 Tick populations

A knowledge of the transmission rate would assist for a better control of TBF in sheep by managerial maintenance of endemic stability through herd immunity. The infection prevalence in ticks is an essential characteristic in the overall transmission rate to sheep but little is known about the prevalence of *E. phagocytophila* in field ticks. Ticks appear to retain infectivity even when feeding on refractory hosts or when they detach from susceptible hosts before they react to the infection (MacLeod, 1936). It is traditionally believed that most of the ticks in the field are infected (MacLeod, 1936; Scott, 1984). The existing estimates confirm the high prevalence of infection with *E. phagocytophila* in ticks (MacLeod, 1936; Webster and Mitchell, 1989; Magnarelli *et al.*, 1995b).

Nymphs can retain infectivity for at least 9 months (MacLeod and Gordon, 1933), the pathogen can persist within the tick for as long as 14 months (MacLeod, 1936). Control of tick-borne diseases could be directed towards reducing tick

populations. Modifying tick natural habitats appears to be the most effective means of control. Suitable habitats for ticks should be identified and modified by farming practice including controlled burning of the vegetation, herbicidal treatment or removal of leaf litter. Pasture improvement will reduce the tick numbers but will also increase the risk for animals when moved to unimproved land. Movement of animals should be avoided, especially pregnant ewes. An endemic stability approach could be tried, thus infection should be ensured in young lambs by means of exposure to a controlled number of infected ticks when they are resistant to clinical signs but become immune to the disease.

Acaricides are widely used in sheep flocks but pesticide resistance appears to occur in ticks with every generation of compound thus emphasising the need for alternative tick control methods. Acaricide treatment should be limited to periods of peak tick activity allowing periodic infestations to ensure continuous challenge of livestock with *E. phagocytophila*. Vegetation treatment with pesticides is best avoided because of the patchy distribution of ticks and sheltered habitats in which they are usually found. Thus large quantities of product and periodic treatments would be required causing environmental pollution and toxic reactions in domestic and wild animals.

2.6.2 Acquired resistance to ticks

Many vertebrate hosts are known to become resistant to tick feeding soon after the first exposure. Sensitised hosts develop a complex immune response involving humoral and cellular components at the site of tick attachment. Basophils play the main role in cell-mediated immunity migrating from the bone marrow to the skin. When they degranulate at the site of tick attachment they stimulate the release of histamine and serotonin, which increase the capillary permeability and induce vasodilatation thus leading to oedema and irritation that provokes the cessation of tick feeding and detachment (Allen, 1991). Langerhans cells of the suprabasal layer of the epidermis collect antigens and present them to T-helper lymphocytes in the regional lymph nodes thus initiating the humoral response. Although antibodies are not the most potent factor in host defence against ticks, some research has

demonstrated that plasma or serum from tick resistant hosts induces variable levels of protection in naive animals (Need *et al.*, 1991). Immune-mediated resistance to ticks is cross-reactive to other ticks of the same or different genera (Labarthe *et al.*, 1985). Tick resistance is known to be heritable in cattle (Allen, 1991) thus tick control can be attained by selective breeding. Host immune responses directly damage tick tissues and physiological processes thus impairing tick engorgement, moulting and reproduction. Synthetic vaccines have been designed against midgut 'concealed' antigens which appear highly protective against tick challenge (Johnston *et al.*, 1986; Kemp *et al.*, 1989) being more effective than vaccination with tick salivary glands homogenates because host antibodies or other compounds lyse epithelial cells and disrupt tick digestion. However, it has been observed that some individuals attempt to repair gut lesions indicating that a resistant strain of ticks may develop.

Immune responses to tick bites have also been observed in humans. Anti-tick saliva antibodies (ATSA) can be measured by ELISA using tick salivary glands as antigen (Schwartz *et al.*, 1993). This method can be used as a biological marker of tick exposure, tick-borne disease risk and to assist in early diagnosis when disease due to a tick-borne pathogen is suspected. There appears to be a direct correlation between tick engorgement index and the amount of saliva injected into the host. Thus individuals with ATSA detectable by the ELISA are more likely to have a tick bite of sufficient duration for disease transmission. On the other hand, studies on rabbits, sheep and cattle to determine their exposure to ticks appear inconclusive (Girardin and Brossard, 1989; Jackson and Opdebeeck, 1989).

2.6.3 Reservoir host populations

Reducing tick numbers by host eradication appears costly and ineffective. Wild deer and rodents from adjacent habitats may quickly repopulate the area unless precluded (Wilson *et al.*, 1988) and once the program is finished the population of ticks is likely to rebound. Deer exclusion by fencing proves to be more effective and appears to be less expensive and labour intensive than acaricide treatments or vegetation management (Bloemer *et al.*, 1990). Livestock exclusion from tick infested areas by means of pasture rotation may help to reduce numbers of *I. ricinus*.

Larger mammals could also be treated regularly or strategically to kill the female ticks responsible for the persistence of tick populations. There appears to be a time lag of 1-2 days between attachment of infected nymphs and infection with *E. phagocytophila* (MacLeod 1936) which may be of relevance for dipping purposes. Brodie *et al.* (1986) suggested to combine dipping of lambs at an early age with a shot of long acting tetracycline as a prophylactic to reduce tick exposure and avoid tick pyemia associated with TBF, responsible for large economic losses in hill areas. The effect of the acaricide will wane gradually thus allowing a progressive exposure to both ticks and TBF.

Control of small vertebrates could be a key point if they prove to be the main reservoirs of infection but it appears very difficult. *Ixodes ricinus* ticks may survive without the presence of sheep in the pasture feeding on birds and rodents for at least 3 years (MacLeod, 1936).

2.6.4 Environmental manipulation

Geographic information systems (GIS) may be useful to identify environmental variables associated with Lyme disease and other tick-borne infections as shown by Glass *et al.* (1995). Nevertheless the extrapolation of risk factors from area to area has to be done carefully because the tick or host behaviour can change radically. For instance, tick life cycles are different in the West and East Coast of Scotland together with the environmental conditions (Curtin *et al.*, 1993). Despite the fact that GIS may be a good indicator of tick distribution in most areas they do not indicate tick abundance. In addition, it is the presence of maintenance hosts for adult ticks what determines their distribution and these hosts are less easy to plot by GIS than vegetation.

2.6.5 Biological control

Biological control is the deliberate introduction of predators, parasites or pathogens to reduce the population of vectors. The same entomopathogenic nematodes (*Steinermatidae*) that have been used for biological control of insect pests of plants appear to impair *Ixodes* ticks survival especially during the last phase of

engorgement (Zhioua *et al.*, 1995; Kocan *et al.*, 1998). Nematodes were able to penetrate tick orifices, death occurring within 24 h of exposure due to septicaemia caused by the symbiont bacteria *Xenorhabdus*. As opposed to insects, nematodes did not reproduce within ticks indicating that repeated applications are required for biological control. *Ixodes dammini* ticks parasitised with the chalcid wasp *Hunterellus hookeri* appear to have reduced viability and, in addition, are not infected with *Borrelia* spirochetes or *Babesia* piroplasms (Mather *et al.*, 1987) thus emphasising the potential of this method for the control of both ticks and tick transmitted diseases.

2.6.6 Chemotherapy and chemoprophylaxis

Most animals will recover without the need of any treatment. Sulphadimidine, tetracyclines and derivatives are the drugs of choice for the clearance of rickettsial organisms from the vertebrate hosts. Shortly after treatment fever is reduced and the parasitaemia is cleared. However, it does not prevent mild relapses which ensures resistance to reinfection. Tetracyclines are particularly useful in concurrent infections because it may also clear Lyme disease spirochetes (Steere, 1989). When secondary infections occur, it might be necessary the use of the appropriate antibiotic depending on each particular case. It is not clear if after antibiotic treatment *Ehrlichia* infection is completely eradicated and/or if the animal remains susceptible. Little is known also about the effects of early therapy on subsequent antibody production.

Canine ehrlichiosis due to *Ehrlichia canis* is also treated with the use of tetracycline hydrochloride or doxycycline hydrochloride, however the clearance of the pathogen is sometimes attained only after months or even years (Wen *et al.*, 1997). This can be due to the persistence of the pathogen in the host or to continuous exposure to the tick vector, which allows reinfection to occur.

Doxycycline and tetracycline induce reduction in fever 24 h after therapy and clear the infection in humans 48h after injection (Dumler and Bakken, 1995). A prompt diagnosis and treatment are essential to prevent complications. Death may occur in some patients if not treated or the treatment starts late due to multi-organ failure associated with progressive ehrlichiosis (Bakken *et al.*, 1994).

2.7 Conclusions

The genus *Ehrlichia* is characterised by being fastidious pathogens difficult to isolate in vivo and in vitro. This has hindered the study of their biological properties and the development of specific diagnostic tests. Recent research has been concentrated in elucidating the genetic relationship between species of the genus thanks to the progress of molecular methods. Little is known, however, about their pathogenic effects within the vertebrate and invertebrate hosts and the mechanisms of alteration of host defences. Increasing research is being currently undertaken to characterise the antigenic properties of monocytic and granulocytic *Ehrlichia*. It is of considerable importance to further demarcate and identify the biological and ecological properties of these and other tick-borne pathogens with similar epidemiology to establish proper strategies towards the reduction of human and veterinary morbidity and mortality.

2.8 Aims of the study

The aims of the study were as follows:

- To determine the rates of infection and distribution of *E. phagocytophila* within the vector, ticks of the genus *Ixodes*, and the vertebrate hosts in widespread sites across Britain

- To identify the major reservoirs of infection for *E. phagocytophila* in the UK, in order to determine the risks posed to humans and livestock populations

- To further characterise several strains of *E. phagocytophila*, including the granulocytic *Ehrlichia* recently isolated from dogs, humans and horses in Europe and North America, by using molecular methods

- To develop highly reliable serological assays for the detection of antibodies to the bacteria in domestic and wild animals

CHAPTER THREE, EPIDEMIOLOGY OF GRANULOCYTIC *EHRLICHIA* PATHOGENS IN SUSPECTED VERTEBRATE RESERVOIRS OF INFECTION

3.1 Evidence of exposure to Ehrlichia phagocytophila *in wild and domestic vertebrate hosts*

3.1.1 Introduction

Ehrlichia phagocytophila is a rickettsial pathogen of domestic sheep and goats (Gordon *et al.*, 1932; MacLeod, 1936, Gray *et al.*, 1988). Cattle are known to be affected by the bacteria although the disease caused in them is usually milder (Hudson, 1950; Foggie and Allison, 1960). Little is known about the ability of wild species of ruminants to be hosts for the organism. Deer and rodents are the main suspected reservoirs for *E. phagocytophila* and the closely related HGE agent (Telford *et al.*, 1996; Belongia *et al.*, 1997; Ogden *et al.*, 1998). It is not known if roe deer only harbour the pathogen and are resistant to clinical signs, or if they also suffer from disease. Early studies showed that, although no intracellular inclusions were found in roe deer blood samples, they appeared infective and reproduced typical signs of TBF infection when experimentally inoculated to susceptible sheep and cattle (MacDiarmid, 1965). Laboratory mice, although showing no or very low bacteremia, are able to sustain transmissible infections of *E. phagocytophila* (Foggie and Hood, 1961). Their wild relatives present reservoir competence under experimental conditions (Telford *et al.*, 1996).

Many species of *Ehrlichia* are pathogenic for dogs, including *E. canis*, *E. platys*, *E. ewingii*, *E. chaffeensis* and *E. equi* (Rikihisa, 1991; Walker and Dumler, 1996). Coinfection with three *Ehrlichia* species has been reported in the same dog (Breitschwerdt *et al.*, 1998) thus explaining serologic cross-reactions with *E. canis* and *E. equi* in the same animal and severe disease manifestations. Experimental infection of dogs with *Cowdria ruminantium* has been achieved, as the animals remained PCR positive for at least two weeks, although no clinical signs were observed (Kelly *et al.*, 1994). *Cowdria ruminantium* immunodominant antigens contain cross-reacting epitopes with *E. canis* that makes the serologic differentiation between the two species very difficult in areas endemic for both pathogens (Matthewman *et al.*, 1993; Matthewman *et al.*, 1994). Atypical cases of monocytic

ehrlichiosis in dogs have been associated with *E. risticii*, a pathogen for horses (Kakoma *et al.*, 1994) indicating that several *Ehrlichia* species can be transmitted to a variety of hosts in nature. There is serological evidence of human and dog exposure to an *E. phagocytophila*-like pathogen in the UK (Sumption *et al.*, 1995; Clark *et al.*, 1996). A HGE-like agent has been isolated from humans, horses and dogs in several parts of Europe such as Sweden, Switzerland and Slovenia (Johansson *et al.*, 1995; Egenvall *et al.*, 1997; Pusterla *et al.*, 1997b; Lotric-Furlan *et al.*, 1998). *Ehrlichia equi* and *E. phagocytophila* have been used as surrogate antigens for the diagnosis of granulocytic *Ehrlichia* in several species. High seroprevalence rates of antibodies to *E. equi* have been found in dogs of some regions of North America (Rodgers *et al.*, 1989) and Europe (Egenvall *et al.*, 1997).

Lewis *et al.* (1975) demonstrated experimental infection of dogs, cats and non-human primates with *E. equi*, a pathogenic granulocytic species of horses in North America, indicating that cross-species transmission is likely. Sheep and goats were also susceptible to *E. equi* infection (Stannard *et al.*, 1969). Antibodies to *Ehrlichia* have been found in feline species of North America and South Africa (Bouloy *et al.*, 1994; Matthewman *et al.*, 1996). The first clinical case of granulocytic ehrlichiosis in a domestic cat has been recently described in Europe (Bjoersdorff *et al.*, 1999). Small mammals also appear to be infected with members of the *E. phagocytophila* genogroup (Walls *et al.*, 1997).

All the evidence suggests that non-ruminant vertebrates including dogs, cats, horses and probably humans are at potential risk for infection with granulocytic *Ehrlichia* because of the ability of the bacteria to enter different hosts and its association with *I. ricinus* ticks, which feed on a wide range of vertebrates.

Perhaps many natural *Ehrlichia* infections go undetected because of the non-specific symptoms leading to misdiagnosis (Petrovec *et al.*, 1997). For instance, a serological survey for the detection of antibodies to *E. equi* in California demonstrated a higher prevalence than expected from the number of diagnosed clinical cases (Madigan *et al.*, 1990). Furthermore, high seroprevalences to *E. canis* (43%) have been found in sera collected from apparently healthy dogs in several areas of Zimbabwe where both *E. canis* and *Rhipicephalus sanguineus* ticks are

endemic. Immunoblot profiles were consistent with an active *E. canis* infection (Matthewman *et al.,* 1993). Those findings suggest a high rate of exposure to a milder strain of *E. canis* or an *E. canis*-like pathogen, which shares antigenic properties. A closely related pathogen, *Cowdria ruminantium,* causes heartwater in ruminants of Zimbabwe but it is believed to be non-pathogenic for dogs. However, *Amblyomma hebraeum* ticks, vector for *C. ruminantium* in the area, can been found feeding on dogs (Norval, 1983). Thus titres to *E. canis* in healthy dogs may only indicate cross-reactivity and exposure to ticks infected with *C. ruminantium* as suggested by some studies (Kelly *et al.,* 1994).

The aim of this study was to investigate if several species of mammals are naturally exposed or infected with granulocytic *Ehrlichia* pathogens in the UK. *Ehrlichia phagocytophila* infected neutrophils were used in an immunofluorescent antibody assay to detect antibodies to granulocytic *Ehrlichia* under the evidence of serologic cross-reactions between species of the genogroup (Dumler *et al.,* 1995). PCR with specific primers for the bacterial cluster were used for the amplification of the pathogen DNA in blood and spleen samples.

3.1.2 Materials and Methods

3.1.2.1 Experimental infection of sheep and preparation of IFA slides with *E. phagocytophila* (Feral Goat isolate) as antigen

Experimental sheep were inoculated with an *E. phagocytophila* stabilate (Feral Goat isolate, see Chapter Seven, Table 7.1). On day three after inoculation sheep showed clinical signs characteristic of TBF infection (Scott, 1984). The proportion of infected cells in the blood was determined by direct counting in Giemsa stained blood smears. A peak of bacteremia was observed on day four, 54% of neutrophils presenting intracytoplasmic morulae. Neutrophils were isolated following a modification of the Carlson and Kaneko (1973) method. Briefly, blood was collected by jugular venipuncture in 10-ml EDTA vacutainers, then centrifuged at 1000 *g* for 15 min. The buffy coat containing monocytes, plasma and the top layer of red blood cells was removed. Three ml of PBS followed by 20 ml of water were

added to lyse the erythrocytes. After 30-45 seconds 10 ml of 2.7% NaCl in phosphate buffer were incorporated into the sample to restore its isotonicity. The whole mixture was centrifuged at 200 *g* for 10 min, then washed twice with PBS, and then centrifuged again for 10 min at 200 *g*. The pellet was resuspended in PBS and the number of cells adjusted to $1x10^7$ cells/ml (Coulter Electronics Ltd.) for use in 15 well slides. Each well was flooded with 15 µl of the final solution. The slides were allowed to dry at 20°C for 30 min, then fixed in acetone for 10 min. They were wrapped in paper and aluminium and stored at -20°C. Before use for IFA the slides were allowed to thaw on the bench at 20°C for at least 15 min.

3.1.2.2 IFA technique

IFA was performed as previously described (Paxton and Scott, 1989) with slight modifications. Serum samples were appropriately diluted, depending on the species, in PBS for IFA (phosphate buffered saline, pH 8.1) and 15 µl dispensed onto each spot. As a negative control, PBS for IFA was used. As a positive control dog sera with known IgG IFA titre (>1/640) was added. Controls for ruminant samples were derived from experimentally inoculated sheep prior and after *E. phagocytophila* infection. Both positive and negative controls were included in each slide.

After an hour in humid chamber the slides were washed 3 times in PBS for 5 min each time. Then aliquots of 15 µl of conjugate were added to the wells and the slides were incubated in humid chamber. After an hour the slides were washed as before. The slides were allowed to dry but not completely and then mounted in 90% glycerol (in PBS) with a coverslip. UV light microscope was employed to detect the fluorescence against the intraneutrophilic morulae of *E. phagocytophila* used as antigen (Fig 3.1). In each well at least 20 neutrophils were examined for each sample. For titration a two-fold series dilution was used, starting from 1/40 (1/100 for roe deer samples). Anti-species immunoglobulin G (IgG whole molecule) FITC (fluorescein isothiocyanate) antibody conjugate (Sigma) was diluted 1/80-1/160 in PBS for use in IFA.

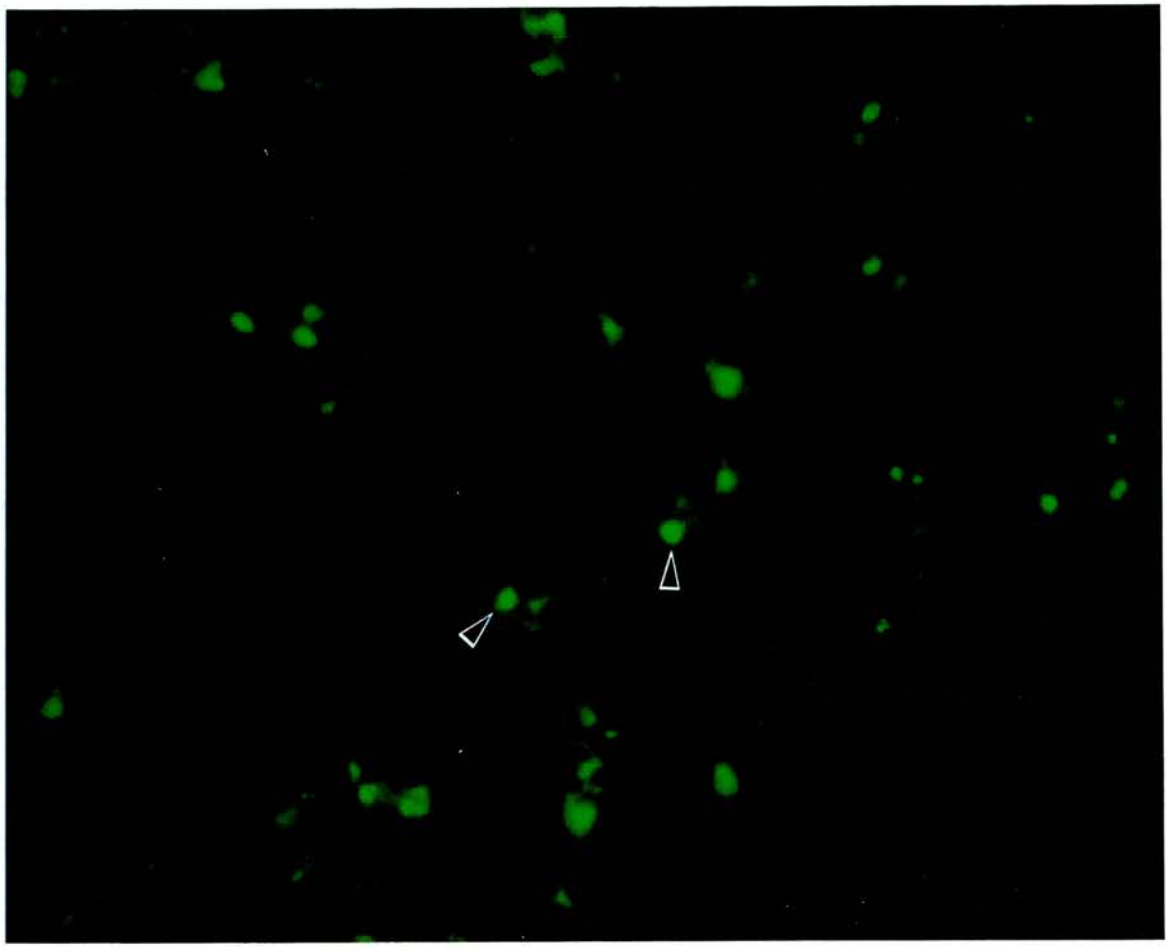


Fig 3.1 IFAT stained neutrophil preparation. Specific antibodies reacted to *E. phagocytophila* intraneutrophilic morulae. Two examples showing the characteristic fluorescence are indicated (arrowheads) (x 650)

3.1.2.3 Serosurvey to determine the presence of antibodies to *E. phagocytophila* in dogs, cats and horses

A random sample of 296 sera from dogs submitted to the diagnostic service (Clinical laboratory) of the Department of Veterinary Clinical Studies of the Royal Dick School of Veterinary Studies in the Veterinary Field Station (Edinburgh) was collected between September and December 1996. Clinical records from the animals were also available. The sample size for estimating the prevalence was determined after Thrusfield (1995). An expected frequency of 50%, a confidence interval of 95% and an error less than or equal to 5% were chosen. Sera were stored at 4°C until analysis and then at -20°C after use. In addition, a sample of 90 dog sera from Caithness (A. Clark, Vet. Invest. Centre, Thurso) was also studied. 76 dog sera from Inverness (SAC Veterinary Services, Inverness) and 27 dog sera from Carmarthen (Veterinary Services, Carmarthen) were included to compare the seroprevalences obtained from the two main areas (Fig 3.2). There was little information available from the last three areas (Caithness, Inverness and Carmarthen) concerning the clinical history of the dogs.

To determine the specificity of the IFA test, 16 serum samples from laboratory reared Beagles without tick exposure (Dr. Bennett, Glasgow University) were examined.

All dog sera starting dilution was 1/40 and the anti-dog conjugate dilution was 1/80 in PBS for IFA, pH 8.1.

For the epidemiological analysis different factors were considered such as sex, age, origin, and size of dog. Data for tick burden or use of dogs were not available. Statistical analyses were performed using the standard computer software Epi-Info Version 6 (Dean *et al.*, 1991), and CIA Version 1.1 (Gardner *et al.*, 1992).

Fig 3.2 Map of Britain showing the origin of canine samples used in serology and the percentage of animals with antibodies to *E. phagocytophila*

3.1.2.4 Collection of samples from wild roe deer (*Capreolus capreolus*)

From November 1997 to November 1998 samples from roe deer (*Capreolus capreolus*) culled during woodland management (thanks to the co-operation of K.A. Urquhart and the British Deer Society) were collected from nine widespread locations in the United Kingdom (Fig 3.3). Serum samples from southern Scotland were also collected from 1994 to 1996. Whole blood in EDTA or spleen for PCR was not available from these deer. Blood was obtained from the iliac vein (Urquhart, 1999) as soon as possible after death of the deer in the field. Blood for PCR was collected in 5 ml EDTA tubes and for serology in 10 ml heparinised tubes. Plasma was harvested from blood in heparin after centrifugation at 1000 *g* for 15 min. A 5g piece of spleen and one foreleg distal to the carpal joint were collected from the same deer and placed in plastic containers. Samples from each deer were transported to the laboratory in separate plastic bags to avoid cross-contamination. All samples were kept frozen at -20°C until use.

Fig 3.3 Map of the UK showing the approximate location of sites where roe deer samples were collected and also the distribution of roe deer across Britain

3.1.2.5 *Ehrlichia phagocytophila* infection in roe deer detected by serology (SDS-PAGE western blot and IFA), PCR and Southern blotting (*groE* gene)

3.1.2.5.1 IFA serology with roe deer plasma and serum samples

Roe deer plasma was harvested from blood in heparin after centrifugation at 1000 *g* for 15 min. Samples were tested for *E. phagocytophila*-reactive antibodies by the indirect immunofluorescence antibody assay (IFA) as described in section 3.1.2.2. Samples were screened at a dilution of 1/100 in PBS, pH 8.1, on spot slides of *E. phagocytophila* (Feral Goat isolate)-infected neutrophils. FITC anti-sheep IgG (whole molecule) conjugate raised in donkey (Sigma) was diluted to 1/80 in PBS. Samples were considered positive when their titres were equal or higher to 1/100. Positive samples were diluted in a 2-fold series and the serologic results were recorded as the highest dilution at which specific fluorescence of *Ehrlichia* morulae could be detected within infected neutrophils. Negative controls were from sheep with no history of TBF and positive controls were from the same sheep after experimental infection with *E. phagocytophila* using blood stabilates.

3.1.2.5.2 SDS-PAGE and Western immunoblotting

IFA results were confirmed using SDS-PAGE of *E. equi* antigen (kindly provided by Dr. Ulrika Munderloh, University of Minnesota) followed by Western blotting (Laemli, 1970), with protocols described in Chapter Six, of weak and strong positive deer sera (with low and high antibody titre by IFA respectively). Briefly, lyophilised antigen was reconstituted in 400 µl of 2x sample buffer. After denaturation at 100°C for 10 min, 100 µl were loaded into each gel and electrophoresed on sodium dodecyl sulphate 10% polyacrylamide (SDS-PAGE) precast minigels (BioRad). Proteins were electrotransferred to nitro-cellulose membranes. After being blocked with 5% skimmed milk in PBS for 1 hour, proteins were reacted with serum at 1/50 dilution in blocking buffer for 1 hour and then by peroxidase-conjugated anti-species IgG at 1/500 dilution in blocking buffer for

another hour. Bands were detected with the colour reagent 4-chloro-1-naphthol. Positive and negative controls were included, derived from experimentally inoculated sheep sera, prior and after infection with *E. phagocytophila*.

3.1.2.5.3 PCR amplification and Southern blotting of *E. phagocytophila groE* gene in roe deer blood and spleen samples

Paired blood (EDTA) and spleen samples were tested by PCR after DNA extraction using a QIAamp Tissue Kit (QIAGEN Ltd) following manufacturers' instructions as described in Chapter Seven. DNA was eluted with distilled water to a final volume of 200 µl for spleen and 100 µl for blood samples. PCR primers HSP534 and HSP1326 were used to amplify a 410-bp fragment of the *groEL* gene from *E. phagocytophila* (Appendix 3.1). For each reaction the PCR mixture contained 0.5 mM Mg Cl_2, 0.2 mM of each dNTP (Boehringer Mannheim), 0.2 µM of each primer, 1.25 units of Taq DNA polymerase (Thermometric Ltd.), plus 5 µl of 10x Ultrotaq buffer (Thermometric Ltd.) and 5 µl of DNA template in a final volume of 50 µl onto which 50 µl of sterile mineral oil (Sigma) was layered. PCR amplification was performed using an Omnigene thermal cycler (Hybaid) with an initial denaturation step at 94°C for 4 min followed by 35 cycles of denaturation at 94°C for 45 sec, annealing at 60°C for 45 sec, and extension at 72°C for 45 sec, followed by a final extension at 72° C for 7 min.

As a positive control, DNA was extracted using a QIAGEN kit from experimentally infected sheep blood when its bacteremia reached 32% as determined after examination under light microscopy of blood smears stained by the Giemsa method.

To confirm the identity of the PCR products, Southern blotting of positive samples followed. An *E. phagocytophila* 410-bp DNA fragment amplified by PCR from experimentally inoculated sheep blood was labelled using a Boehringer Mannheim labelling and detection kit following manufacturers' instructions. Briefly, 1 µl of the PCR product from the positive control was diluted with sterile distilled water to a final volume of 15 µl. DNA was denatured by boiling for 10 min. Then 2

µl of hexanucleotide mixture (10x), 2 µl of dNTP labelling (mixture), and 1 µl of Klenow enzyme, labelling grade, final concentration 100 U/ml, were added and mixed. The sample was incubated overnight at 37°C, then 2 µl of 200mM EDTA, pH 8.0, were added to stop the labelling reaction and the samples stored at -20°C until use.

PCR positive samples from blood and spleen were electrophoresed through 1% agarose gels at 70 V in TBE buffer (89 mM Tris-borate, 2.5 mM EDTA) for 30 min. Five ml of a 1 Kb ladder (Gibco) were included in the gel to confirm the PCR product size. Agarose gels were recovered and assembled into a sandwich for blotting to positively charged Nylon membranes Hybond^{TM}-N+ (Amersham International) using a Transblot (SD semi-dry electrophoretic cell (BioRad) following manufacturers' instructions. DNA was transferred to the membrane at a constant current of 3.55 mA/cm2 for 10 min. Following transfer the membranes were recovered and the DNA fixed with 0.2 M NaOH, then the membrane was baked at 80°C for 30 min. Hybridisation was carried out as described in the Hybridisation Protocol booklet supplied by Boehringer Mannheim and summarised in Chapter Four.

3.1.2.6 Detection of antibodies to *E. phagocytophila* and PCR amplification of ehrlichial DNA (16S rRNA gene) from cattle and sheep plasma and sera after an abortion outbreak

An abortion outbreak in a flock of sheep in Penrith was suspected to be associated with *E. phagocytophila* infection. Twelve sheep sera were submitted (Phil Watson, Vet. Invest. Centre, Penrith) to the CTVM to confirm the diagnosis. Six further samples were submitted for analyses from a different abortion flock. In addition 8 cattle samples from an abortion outbreak in Penrith were also included.

DNA from the samples was extracted as follows. Sheep sera was centrifuged for 15 min at 18000 *g*. The supernatant was removed and the pellet resuspended in 25 µl of lysis buffer containing 10 mM Tris, 1 mM EDTA and 1% Tween 20. 200 ng of Proteinase K were added to the buffer and the solution was heated at 55°C for an

hour, then boiled for 10 min to inactivate the enzyme and stored at 4°C until use. 5 µl of the sample where used as a template directly in PCR reactions.

For each reaction the PCR mixture contained 0.5 mM Mg Cl_2, 0.2 mM of each dNTP (Boehringer Mannheim), 0.2 µM of each primer, 1.25 units of Taq DNA polymerase (Thermometric Ltd.), plus 5 µl of 10x Ultrotaq buffer (Thermometric Ltd.) and 5 µl of DNA template in a final volume of 50 µl onto which 50 µl of sterile mineral oil (Sigma) was layered. PCR amplification was performed using an Omnigene thermal cycler (Hybaid) with an initial denaturation step at 95°C for 5 min followed by 40 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 1 min, and extension at 72°C for 1 min, followed by a final extension at 72°C for 7 min. The annealing temperature was lowered to 50°C in the second round of amplification. PCR primers 856-1154 in the outer amplification and GER3-GER4 (Munderloh *et al.*, 1996b) for the inner amplification were used (Fig 3.10; Appendix 3.1).

IFA protocol was performed as previously described in section 3.1.2.2 using anti-species, sheep or cattle, FITC conjugates (Sigma) at 1/80 and 1/160 dilutions respectively in PBS.

3.1.3 Results

3.1.3.1 Evidence of exposure to *E. phagocytophila* in dog, cat and horse populations in the UK

3.1.3.1.1 Serological response to *Ehrlichia phagocytophila* antigen

Sera from all 16 laboratory reared experimental dogs from Glasgow gave negative results at a titre of 1/40. Titres of 1/40 and above were considered positive. 12/293 (4%) dog sera from Edinburgh area were positive with titres ranging from 1/40 to 1/160 (Table 3.1). Three cat sera were inadvertently included in the random sample of dogs. When analysed (using anti-cat FITC conjugate, Sigma, 1/80 dilution in PBS) one of them was positive with a titre of at least 1/160.

Sera from Caithness produced 16/90 (18%) positive animals. Very little information was available from the clinical records of the dogs of that area. Five cats and eighteen horses were also submitted for serological analysis. 3/5 cats and 4/18 horses were seropositive. Although the seroprevalences appear to be high for those species, especially for cats, a higher sample size is necessary before making further conclusions.

Table 3.1 IFA titres of canine samples positive to *E. phagocytophila,* Feral Goat isolate, in the two main areas, Edinburgh and Caithness. Differences in titre between the areas was not statistically significant

	Proportion (%)		Uncorrected χ^2	
IFA titre (IgG)	Edinburgh	Caithness	χ^2	p
1/40	8/12 (66.7)	5/9 (56)	0.27	>0.05
1/80	1/12 (8.33)	4/9 (44)	3.7	>0.05
1/160	3/12 (25)	0/7 (0)	2.29	>0.05

A sample of 27 dogs from Wales (Carmarthen) gave a similar rate to the obtained from Caithness (18%), thus suggesting a high seroprevalence in rural dogs.

From Inverness 7/76 dogs showed to be seropositive, giving a higher seroprevalence (9%) but similar to that obtained from Edinburgh. A sample of nine horses produced higher seroprevalences, 7/9 animals were positive. That difference in seroprevalence between species of the same area could be explained because of the different exposure to ticks in pet dogs and horses.

3.1.3.1.2 Clinical features of the positive dogs

Table 3.2 summarises some of the clinical signs and chemistry records found in positive dogs on the day of sera collection. Most of the seropositive animals showed high liver enzyme levels, neutrophilia, and high white blood cell counts. When compared to the seronegative animals the differences were not statistically significant. That can be explained because dog sampling was made from a population of animals that were already ill.

Table 3.2 Summary of clinical signs and chemistry in positive animals of (a) Edinburgh and (b) Caithness

(a) Edinburgh

Animal No.	Sex	Age	Clinical signs	IU/l ALT	AP	µmol/l Creatinine	mmol/l Urea	x10^9/l WBC	% N	% L	g/l T. prot.
19	M	2	Complaint	40	74*	42	3.5	9.5	70	14	62
53 (cat)	N	8	Complaint [1]	68*	na	720*	48.1*	na	na	na	na
92	M	11	Complaint [2]	24	20	119*	2.6	11	72	18	65.5
105	M	5	na	58	98*	1252*	122*	21.1*	92*	3*	38.3*
111	M	12.5	Ear pain	49	30	75	4	10.9	64	21	62.4
118	M	10	na	62*	187*	71	3.8	17.9*	90*	3*	62.5
129	F	8	Anorexia, ascites	38	280*	113*	18*	34.4*	85*	8*	55.9*
142	F	12	Chronic hypertension	33	84*	139*	13.6*	12.2	81*	11	64.8
161	M	<1	Complaint	40	162*	81	7	27*	70	13	54.7*
198	na	11	Complaint	33	749*	64	4.8	12.4	84*	6*	75.6*
236	M	11	Ascites	77*	284*	66	7.2	16.7*	73	24	43.5*
250	na	na	na	na	na	na	na	na	na	na	na
Mean		8.35		45.40	196.80	561.90	28.75	17.31	78.10	12.10	58.52
Median		10.5		40	130	651	10.40	14.55	77	12	62.20
SD		4.22		16.07	215.36	366.47	39.38	8.12	9.54	7.31	10.95
Range		<1-12.5		24-77	20-749	42-1252	3.5-122	9.5-34.4	64-92	3-24	38.3-75.6
Reference interval				15-60	20-60	30-90	1.7-7.4	6-15	40-80	10-36	58-73

* Out of the normal range value

[1] Weight loss, polydipsia, mouth ulceration

[2] Laryngeal paralysis

na data not available

AP Alkaline phosphatase

ALT Alanine aminotransferase

WBC Total white blood cell count

N Neutrophils

L Lymphocytes

T. prot Total protein

(b) Caithness

Animal No.	Sex	Age	Clinical signs	WBC ($x10^9/l$)
M556[1]	na	na	lethargy	25*
M503[2]	na	8	lethargy	na
M534[3]	F	na	pyrexia	42.5*

* Out of the normal range value

1 Dog showed pyrexia, lymphopenia, neck pain, and lethargy 3 weeks post tick exposure. An autoimmune polyarthritic disorder was suspected. The dog recovered gradually after 4 weeks.

2 The dog was exercised in a tick infested wood

3 The dog showed suddenly off colour. A short period later the dog underwent hysterectomy for pyometra.

na data not available

3.1.3.1.3 Epidemiological analysis

Table 3.3 summarises the differences in seroprevalence for the four areas studied. Edinburgh and Inverness showing the lowest seroprevalences, Caithness (Scotland) and Carmarthen (Wales) having similar seroprevalences (Fig 3.2). Although the difference in seroprevalence between Edinburgh and Carmarthen is statistically significant (data not shown), the 95% confidence intervals overlap, thus emphasising the need for further testing. Edinburgh and Inverness showed lower rates of exposure to *E. phagocytophila* in dogs. When analysed, the difference in seroprevalence was not statistically significant and the 95% confidence intervals did overlap thus suggesting a similar rate of infection.

Table 3.4 indicates the differences in seroprevalence in horses and cats from Caithness and Inverness. The small sample sizes do not allow us to draw further conclusions.

The 95% CI for the two main areas Edinburgh and Caithness are represented in Fig 3.4. The 95% CI for sex, age, and origin (Edinburgh) shown in table 3.5 overlap and they are not graphically represented. Age and sex showed no significant differences although 77% (7/9) of the animals in the positive group were males and 22% (2/9) were females whereas for the whole population 57% were male and 43% were female.

Epidemiological findings of positive dogs to the *E. phagocytophila*-like agent in dogs of the Edinburgh area are summarised in table 3.6. Of the positive animals 50% showed neutrophilia N>80 (5/10) and 80% had AP>60 IU/l. 40% presented lymphocytopenia L<10 (4/10). There was a wide variation in the biochemical parameters when considering the negative animals. Table 3.7 shows a comparison in the differences in prevalence for some of the features found in both positive and negative dogs to *E. phagocytophila*.

Positive dogs belonged to five different breeds, one Welsh springer spaniel, one German shepherd dog, one small Poodle, one Labrador, and two Cocker spaniels. Two more dogs were crosses and it was not possible to determine their size. The cat was a domestic short-haired. Data from the rest of the animals were not available.

Table 3.3 Differences in prevalence of antibodies to *E. phagocytophila* in canine samples according to area

Area	Proportion[a]	P[b]	95% CI[c]	χ^2	p		95% CI[d]
Edinburgh	12/293	4 %	2.13, 7.04	17.06[e]	<0.01	S	0.05-0.22
Caithness	16/90	18 %	10.52, 27.26	21.37[f]	<0.01	S	
Inverness	7/76	9 %	3.78, 18.06				
Carmarthen	5/27	18 %	6.3, 38.08				

[a] No. positive/No. sampled

[b] Seroprevalence (point estimate)

[c] Exact binomial 95% confidence intervals

[d] 95% confidence intervals for the difference between proportions (Edinburgh-Caithness). The difference does not include 0 and is therefore significant

[e] Edinburgh-Caithness

[f] All four areas

S Statistically significant (Yates corrected)

Table 3.4 Differences in prevalence to *E. phagocytophila* depending on the species in Caithness and Inverness

Species	Proportion[a]	P[b]	95 % CI[c]
		Caithness	
Horses	4/18	22 %	6.41, 47.64
Cats	2/5	40 %	5.27, 85.34
		Inverness	
Horses	7/9	78 %	40.0, 97.19

[a] No. positive/No. sampled

[b] Seroprevalence

[c] Exact binomial 95% confidence intervals

Table 3.5 Differences in prevalence to *E. phagocytophila* according to sex, age and origin in dogs within Edinburgh area

Sex	Proportion	P[a]	95% CI[b]	χ^2	p		95 % CI[c]
Male	7/140	5%	2.03, 10.03	0.89	>0.05	NS	-0.01, 0.08
Female	2/106	1.89%	0.23, 6.65				
Age							
< 3 years	2/44	4.56 %	0.55, 15.47	1.48	>0.05	NS	
3-7 years	1/69	1.45 %	0.04, 7.81				
> 7 years	8/168	4.76 %	2.08, 9.17				
Origin							
Edinburgh	9/243	3.7 %	1.7, 6.91	0.13	>0.05	NS	-0.09, 0.05
Rest	3/50	6 %	1.25, 16.55				

a Seroprevalence (point estimate)
b Exact binomial 95 % confidence intervals
c 95 % confidence intervals estimates for the difference between proportions according to sex and origin. It includes 0, indicating no significant difference in prevalence
NS Not statistically significant (Yates correction, Fisher exact 2-tailed)

Table 3.6 Epidemiological features of dogs positive to *E. phagocytophila* antigen in the Edinburgh area

	Proportion of animals	%
Alkaline Phosphatase >60 IU/l	8/10	80
WBC >15x10^9/l	5/10	50
Neutrophilia (%N>80)	5/10	50
Creatinine > 90 mmol/l	5/11	45
Total protein <58 g/l	4/10	40
Lymphocytopenia (%L<10)	4/10	40
Male	7/9	77.7
> 7 years old	8/11	73
Size of dogs	Medium-large	

WBC Total white blood cell count
N Neutrophils

Table 3.7 Comparison of some of the features most relevant in dog samples from Edinburgh that appeared positive and negative to *E. phagocytophila* by IFA

	Negative		Positive				
	Proportion	%	Proportion	%	χ^2	p	
AP > 60 IU/l	154/234	65.8	8/10	80	0.35	>0.05	NS
WBC >15x10^9/l	87/230	37.8	5/10	50	0.2	>0.05	NS
Total protein <58 g/l	69/230	30	4/10	40	0.1	>0.05	NS
Lymphocytopenia	90/230	39.1	4/10	40	0.08	>0.05	NS
Male	133/236	56.4	7/9	77.7	0.87	>0.05	NS
>7 years	160/271	59	8/11	73	0.35	>0.05	NS

AP Alkaline phosphatase
WBC Total white blood cell count
NS Not statistically significant (Yates corrected; Fisher exact 2-tailed p value)

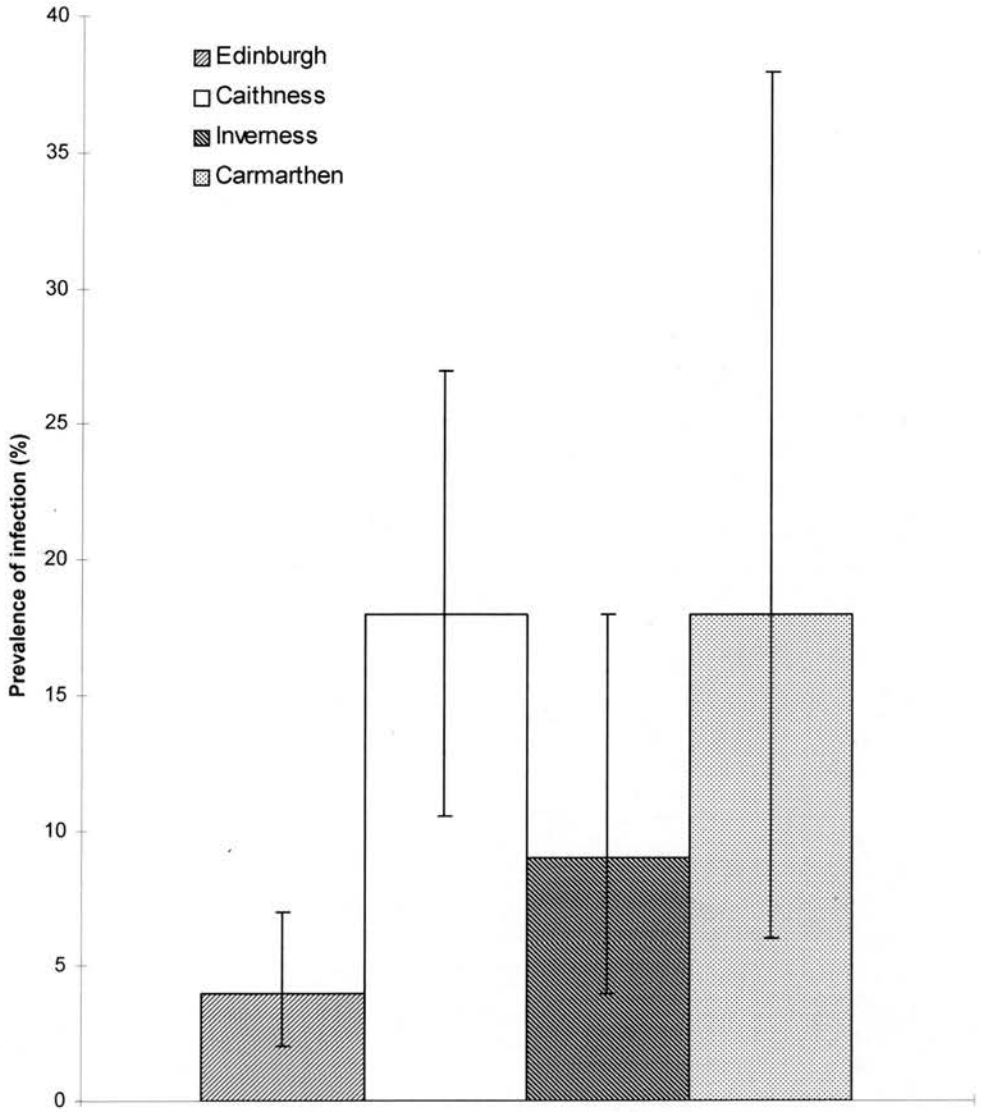


Fig 3.4 Exact binomial 95% Confidence Intervals (CI) between the four areas represented by the Y error bars. Edinburgh and Caithness 95% CI did not overlap indicating a true difference in prevalence

3.1.3.2 Identification of roe deer as competent reservoirs for *E. phagocytophila* by means of serology and PCR

3.1.3.2.1 Collection of roe deer samples

In total, samples were collected from 104 roe deer. These included 102 plasma/serum samples, 82 pieces of spleen, 71 legs, and 84 blood in EDTA tubes. Eight samples including the leg were paired to the right ear from the same animal. Table 3.8 shows the number of animals sampled from each site according to sex and age.

No information was available regarding the body condition or general health status of the animals.

Buffy coat smears from some animals were Giemsa stained to detect the presence of the organism. Unfortunately the quality of the samples was not suitable for adequate microscopic examination and assessment of infection.

Table 3.8 Number of roe deer samples collected from each site separated according to sex and age

	Sex		Age	
Site Number	Male	Female	Adult	Calf
1	1	0	1	0
2	10	8	13	5
3	1	1	2	0
4	15	19	20	14
5	2	1	2	1
6[a]	14	6	13	4
7	3	5	7	1
8	10	3	10	3
9	1	0	1	0
Total	57/100	43/100	69/97	28/97

[a] Discrepancies in the number of animals are due to lack of details from the sample

3.1.3.2.2 Serology with roe deer plasma and serum samples

A total of 102 plasma/serum samples were tested by IFA. 59 had *E. phagocytophila*-reactive antibody titres of 1/100 or higher giving an overall prevalence of 58%. Site specific antibody prevalences are shown in Table 3.9. According to age or sex no significant differences in seroprevalence were found (Table 3.10).

A significant difference ($\chi^2 = 32.73$; $p<0.001$) in seroprevalence was found between site 4 and four other sites (2, 6, 7, and 8). The seroprevalence in these regions was significantly higher than at site 4 when the confidence intervals for the point prevalences were considered (Table 3.9, Fig 3.5) but they overlapped when site 4 was compared with site 6. This indicated high variation in prevalence of infection between sites. The small sample size from sites 1, 3, and 5 does not permit further conclusions. The high seroprevalence corresponded positively with higher percentages of PCR positive for *E. phagocytophila* in blood and spleen, as shown in tables 3.9 and 3.12. As for individual animals the seropositivity did not always correspond to PCR positive samples either in blood or spleen although 80% of the PCR positive animals were also positive by IFA and 60% of the seropositive animals were also positive by PCR (Table 3.11). These results can be explained because this was a cross-sectional study and deer may be at different stages of *Ehrlichia* infection and subsequent antibody response. PCR positive deer were probably more recently infected.

Western blot analysis of IFA positive roe deer samples confirmed their exposure to an *E. phagocytophila*-like pathogen. The samples, including the positive control, reacted to a 44-kDa protein, specific for granulocytic *Ehrlichia* (Dumler *et al.*, 1995; Wong *et al.*, 1997; Ravyn *et al.*, 1998) (Fig 3.6).

Table 3.9 Summary of serologic tests, PCR assays, and Southern blot for *E. phagocytophila* in roe deer samples from nine widespread sites in the UK

	Serology (IFA)				PCR assays (No. of roe deer positive/total no.tested)		Southern blot (No. of roe deer positive/total no.tested)	
Site	No. of roe deer with titres of ≥ 100/total no. tested	95% CI[a]	Geometric mean titre[b]	Maximum titre	Blood	Spleen	Blood	Spleen
1	0/1	0-98	-	-	0/1	0/1	0/1	0/1
2	15/18*	59-96	2918	12800	14/18	10/18	14/18	9/18
3	0/2	0-84	-	-	0/2	0/2	0/2	0/2
4	10/38*	13-43	230	6400	0/18	1/16	0/18	1/16
5	3/3	29-100	1600	3200	0/3	1/3	0/3	1/3
6	11/18*	36-83	1096	3200	9/20	5/20	7/20	5/20
7	8/8*	63-100	1745	3200	5/8	5/8	5/8	3/8
8	12/13*	64-99	2851	12800	6/13	3/13	6/13	3/13
9	0/1	0-98	-	-	0/1	0/1	0/1	0/1
All	59/102	-	1423	12800	34/84	27/82	32/84	24/82

[a] Exact binomial 95% Confidence Intervals calculated using EPI6 v.6.04

[b] Data for samples negative at 1/100 dilution were omitted

* Differences in seroprevalence between the five sites were statistically significant, $\chi^2_{df=4} = 32.73$; $p<0.001$

Key for sites: 1, Balblair; 2, Auchtertyre; 3, Dundas; 4, Borders; 5, Kirkhouse; 6, Kyloe; 7, Moncreiffe; 8, Euston; 9, Whytham

Table 3.10 Prevalence of antibodies to *E. phagocytophila* in roe deer samples separated according to sex and age. Differences in seroprevalence were not statistically significant (p>0.05)

	Sex			Age		
IFA	Male	Female	Total	Adult	Calf	Total
+	32	25	57	42	13	55
-	24	17	41	25	15	40
	56	42	98	67	28	95
χ^2	0.06			1.53		
p	> 0.05			> 0.05		

Table 3.11. Roe deer samples (either blood or spleen) that appeared positive by PCR showing a fragment of the expected size (410 bp) from the *groE* operon, were compared to the number of animals that exhibited reactivity by IFAT to *E. phagocytophila*. The difference was statistically significant and Kappa coefficient indicated fair agreement between tests

	PCR (blood or spleen)		
IFA	+	-	
+	33	22	55
-	8	19	27
Total	41	41	82

Yates corrected	χ2 df=1= 5.52; p<0.05
Kappa coefficient	0.27
Observed agreement	0.63
Chance expected agreement	0.50
Standard error of Kappa	0.10
Z	2.58
One-tailed p-value	0.0049

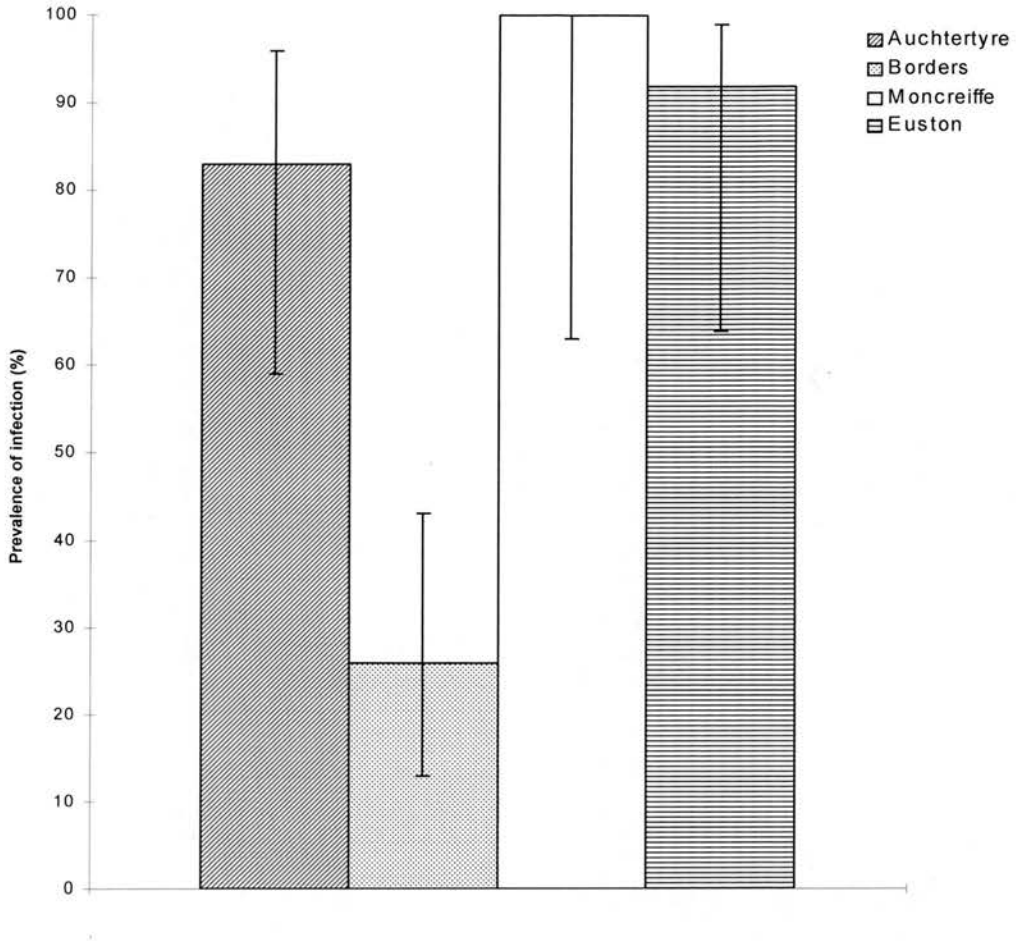


Fig 3.5 Seroprevalence by IFA and 95% Confidence Intervals (CI), represented by the Y error bars, of different roe deer populations in four widespread collection sites. Confidence intervals between Borders and the rest of the sites do not overlap indicating there is a true difference in prevalence between those areas

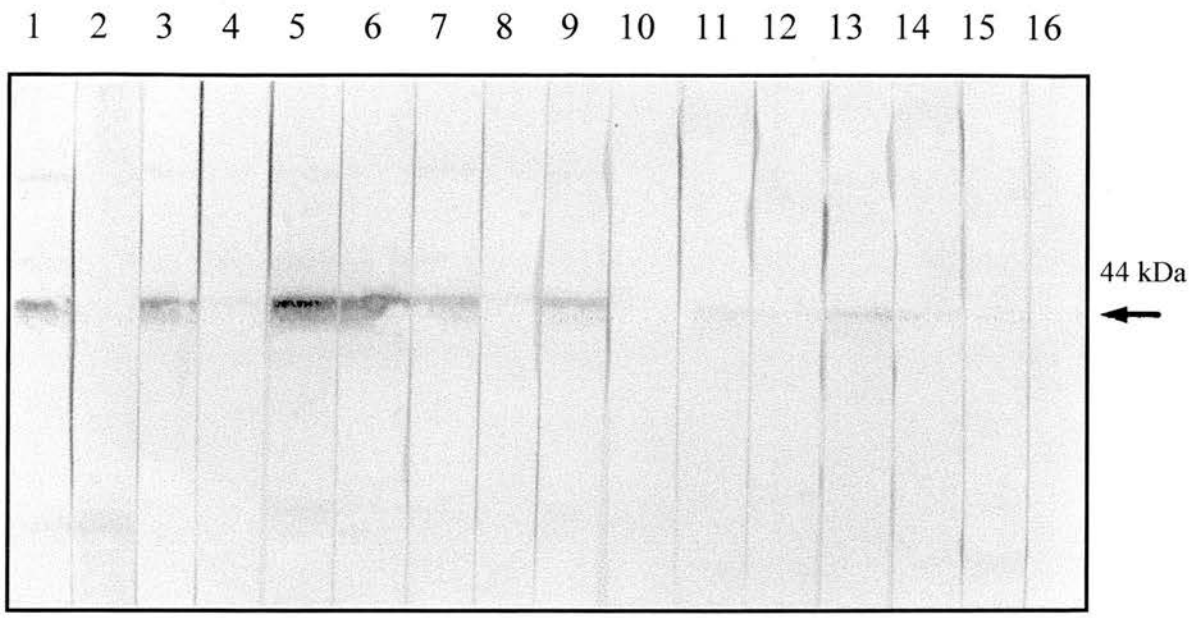


Fig 3.6 Western immunoblot of several roe deer samples with high (lanes 3 to 9) and low titres (lanes 10 to 16) by IFA with *E. phagocytophila* as antigen. *Ehrlichia equi* proteins were separated with a discontinuous (4-10%) PAGE, then they were transferred to nitro-cellulose membranes and incubated with 1/100 dilutions of antiserum. Lane1, serum from sheep experimentally inoculated with *E. phagocytophila*, 3 weeks after infection; Lane 2, serum from the same sheep prior to inoculation. Positive samples, including the control, reacted to a 44-kDa protein (arrow), specific to granulocytic *Ehrlichia*

3.1.3.2.3 Amplification of *E. phagocytophila* DNA in roe deer blood and spleen samples

PCR specific primers amplified a 410-bp product (Fig 3.7) from *E. phagocytophila* DNA but they did not amplify DNA from the closely related species *Cowdria ruminantium*, *E. bovis* and *E. canis*. Interestingly, DNA from another granulocytic *Ehrlichia*, *E. ewingii*, was amplified after PCR but the organism has been only isolated from dogs in the United States and will not interfere with the study.

Two PCR products derived from blood and three derived from spleen appeared to be a few base pairs smaller than the controls. Southern blotting was performed to confirm the specificity of the PCR product. The samples that appeared to be slightly smaller were not recognised by the probe and they were considered negative (Fig 3.7.b). Overall 32 (38 %) of blood and 24 (29 %) of spleen samples were positive by PCR as confirmed by Southern blotting (Table 3.9).

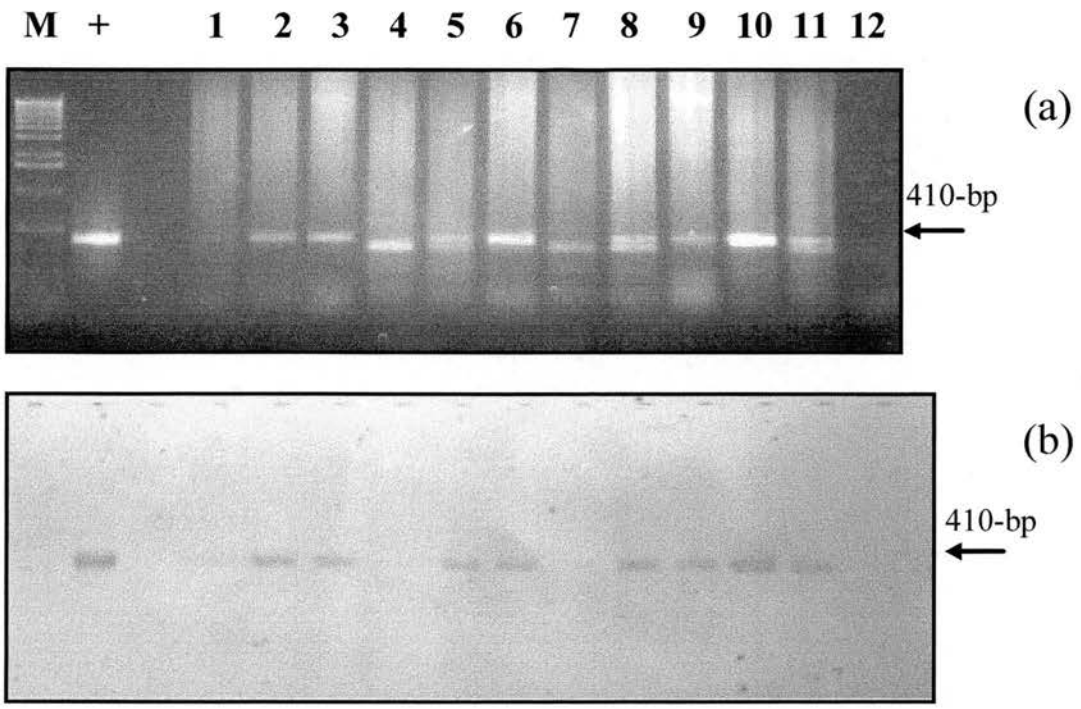


Fig 3.7 (a) PCR using specific primers for the *groE* gene of granulocytic *Ehrlichia* for roe deer spleen samples (b) Southern blot of the same samples. A 410-bp fragment obtained from sheep blood experimentally infected with *E. phagocytophila* was labelled and used as a probe. Arrows indicate the position of the 410-bp band specific for granulocytic *Ehrlichia*. Lane +, blood from experimentally infected sheep; Lanes 1-11 roe deer spleen samples; Lane 12, sterile distilled water. Samples in lanes 4 and 7, which appeared slightly smaller in the agarose gel, were not recognised by the probe

3.1.3.3 Evidence of infection with *Ehrlichia phagocytophila* associated with abortion outbreaks in sheep and cattle

All 12 sheep sera tested from the first outbreak were positive at 1/40 dilution, whereas only 8/12 were positive at 1/200 dilution in PBS by IFA. A fragment of 151-bp from the 16S rDNA sequence of *E. phagocytophila* was successfully amplified from two sheep sera after nested PCR. One product that appeared bigger than expected was considered negative.

In the second outbreak 4/6 animals were positive when tested by IFA at 1/200 dilution. By nested PCR two more samples were positive, one of them did not correspond to an IFA positive result (Table 3.12). Foetal fluid from one of the aborted sheep appeared negative by IFA and nested PCR.

All cattle samples were positive by IFA at 1/200 dilution and 6/8 were positive after nested PCR.

Table 3.12 Summary of serologic and PCR results for sheep samples associated with an abortion outbreak in Penrith

First outbreak			Second outbreak		
Sheep No.	IFA	PCR	Sheep No.	IFA	PCR
1	+	-	1	+	+
2	+	-	2	-	-
3	+	-	3	+	-
4	+	-	4	-	+
5	+	+	5	+	-
6	+	-	6	+	-
944	-	-			
945	-	-			
946	+	-			
947	+	+			
948	-	-			
949	-	-			

3.1.4 Discussion

3.1.4.1 Serological results in a canine population

Each of the experimental Beagles from Glasgow reared under tick free conditions gave negative results by IFA at 1/40 titre, thus suggesting the test has a high specificity.

The seroprevalence in Edinburgh appeared to be very low. This prevalence when compared with that obtained in Caithness was statistically significant (χ^2=17.06, p<0.01). Although for a comparison in tick burden between areas data were not available, higher tick exposure in rural areas can be presumed. Transmission for the newly discovered granulocytic *Ehrlichia* isolates is not confirmed yet, but tick vectors are suspected as for the closely related species *E. equi* (Richter *et al.*, 1995) and *E. phagocytophila* (MacLeod and Gordon, 1933). In addition, Telford *et al.* (1996) have demonstrated vector competence of *I. dammini* for the HGE agent in a laboratory rodent model.

Previous research has shown higher titres to *E. canis* in cases of canine granulocytic ehrlichiosis than to *E. equi* with negative serologic results for other *Ehrlichia* species (Stockham *et al.*, 1992). That *E. canis* 'milder strain' was later on described as *E. ewingii* (Anderson *et al.*, 1992). It is genetically more closely related to *E. canis* than to the *E. phagocytophila*-like agents and it is transmitted by *Amblyomma* ticks that are not present in northern Europe. Neither *E. canis* nor *E. ewingii* are expected to be present in UK and serologic reactions are therefore presumed only to be for granulocytic *Ehrlichia* species. Therefore titres in this experiment are probably due to an *E. phagocytophila*-like agent.

Antigen heterogeneity has been observed for the different isolates of *Ehrlichia phagocytophila* in cattle and sheep (Foggie and Allison, 1960; Woldehiwet and Scott, 1982b). A question arises about the antigen used for our IFA test because the titres we obtained were very low. Since different strains of *E. phagocytophila* do not appear to give full protection against the heterologous challenge, the antigenicity of the strain used for diagnosis in the study could differ significantly from the isolate

affecting dogs. If the agent causing canine granulocytic ehrlichiosis is similar to the human agent in the US greater titres to the homologous antigen are expected. In Chapter Seven it is demonstrated that cases of granulocytic ehrlichiosis in European dogs and horses differ from both the HGE agent in the United States and *E. phagocytophila* in Europe at *groE* gene level although they appear more closely related to the latter. The use of *E. phagocytophila* as a surrogate antigen may be missing some of the animals with antibodies to granulocytic *Ehrlichia* because different species of bacteria may not share all immunodominant antigens. Titres to granulocytic *Ehrlichia* are known to vary depending on the type of antigen used. Titres are usually higher to *E. equi* than to *E. phagocytophila* in human cases of granulocytic ehrlichiosis in North America and some of the patients positive for *E. equi* were even negative for *E. phagocytophila* (Bakken *et al.,* 1994; Dumler and Bakken, 1995). Furthermore, antigenicities and pathogenicities of HGE bacterial strains are known to vary among geographic regions (Bakken *et al.,* 1996).

3.1.4.2 Clinical and epidemiological findings

Clinical signs associated with canine granulocytic ehrlichiosis due to *E. ewingii* (Stockham *et al.,* 1992) are an acute polyarthritic disorder involving lameness and muscular stiffness. Despite the cell preference, *E. ewingii* is genetically and serologically more closely related to *E. canis* than to the *E. phagocytophila* genogroup (Stockham *et al.,* 1992; Anderson *et al.,* 1992). In this study, dogs showed a variety of clinical disorders including musculoskeletal involvement. The commonest signs were seizures, general complaint, lethargy, and pyrexia. Alterations of biochemical parameters were also found such as raised liver enzymes which appears to be characteristic in HGE infections (Bakken *et al.,* 1996). Thrombocytopenia is commonly reported in many rickettsial infections including *E. canis*, infectious cyclic thrombocytopenia associated with *E. platys* (Harvey *et al.,* 1978) and Rocky Mountain spotted fever (Greene *et al.,* 1985). No data were available regarding the platelet count for this study but thrombocytopenia has been found to occur in dogs with suspected granulocytic *Ehrlichia* infection in Scotland (Clark *et al.,* 1996).

There seemed to be a preference for male, large-medium, older than 7 years old dogs. In other studies there does not appear to be any sex preference (Egenvall *et al.*, 1997) but they are in agreement with findings in relation to size of dogs and age. Bigger dogs are more likely to expend more time in outdoor activities and they can also carry more ticks. Older dogs are more likely to seroconvert because of the higher potential of tick and pathogens exposure. Younger dogs may have maternal immunity and be resistant to overt clinical signs of disease as occurs with young lambs (Stuen, 1993). Clinical response to TBF appears to be more severe with increasing age of lambs, however, young animals also mount an immune response towards the organism. It is therefore likely that higher titres are found in older dogs after repeated exposure to the pathogen.

Three cat sera were inadvertently included in the sample, one of them appearing to be positive by IFA. Cat sera are known to react with antigens of *Ehrlichia canis* (Matthewman *et al.*, 1996). High seroprevalences to *Ehrlichia* species have been found in felines of the United States (Bouloy *et al.*, 1994). Experimental infection with *E. equi* has been attained in cats (Lewis *et al.*, 1975) although the induced disease was mild and the pathogen was observed only in eosinophils. The role of cats and particularly wild cats in the epidemiology of *Ehrlichia* associated diseases may have been overlooked. Recent studies have identified the first clinical case of feline granulocytic ehrlichiosis in Europe (Bjoersdorff *et al.*, 1999) suggesting cats can be hosts for the bacteria. Cats may be exposed to arthropods that pick up infection on mice, they may even acquire the pathogen after ingestion of infected hosts. There is some recent evidence suggesting that oral transmission from the dam to the calves is possible (Pusterla *et al.*, 1998a). Rodents seem to play a role in the maintenance of other diseases such as babesiosis or Lyme borreliosis, and they can also harbour the agent of granulocytic ehrlichiosis (Magnarelli *et al.*, 1997, Ogden *et al.*, 1998). High seroprevalences to *E. phagocytophila* were found in this study in horses and cats suggesting their involvement as reservoirs of infection for *E. phagocytophila*-like organisms. Any animal bitten by *Ehrlichia* infected ticks would have the potential to serve as reservoir, although it is more likely that cats and horses are aberrant hosts for the

bacteria because, at least pet animals, they do not appear to support large tick infestations.

Despite the small sample size obtained from Caithness and the consequent wider confidence interval, it already shows that the true prevalence in the area does not overlap with the one obtained from Edinburgh. The difference was also statistically significant. A higher seroprevalence to a *E. phagocytophila*-like agent in dogs, and also humans and horses, may be expected in rural areas endemic for *Ixodes* ticks. Naive dogs may undergo disease if they are exposed for the first time to ticks infected with the granulocytic pathogen in rural areas. Recent studies have indicated a low seroprevalence to tick-borne zoonoses, including HGE infection (Ag used?), in farmers of England suggesting that, although uncommon, there is human exposure to *Borrelia* and *Ehrlichia* species in rural areas (Thomas *et al.*, 1998).

Sheep are known to carry *E. phagocytophila* for long periods (Foggie, 1951). It is believed that ticks can pick up infection during carrier state as well as during patent bacteremia. If tick transmission for the new human and animal isolates is confirmed and also the ability of the pathogen to remain infective for ticks in the vertebrate hosts during long periods, the risk of acquiring infection might be further increased. In addition, there is strong evidence to support the zoonotic potential of the isolates. It appears that granulocytic *Ehrlichia* can adapt to different hosts other than ruminants and even produce similar clinical signs (Engvall *et al.*, 1996). Dogs are known to be persistently infected with *E. canis*, as detected by PCR, for months even after antibiotic treatment (Wen *et al.*, 1997; Breitschwerdt *et al.*, 1998) which may also indicate continuous re-exposure to ticks carrying the pathogen.

Further research is necessary to elucidate the role of dogs, horses, and cats in the epidemiology of the disease and as potential reservoirs for human infection.

3.1.4.3 Role of roe deer as competent reservoirs for *E. phagocytophila*

In the United States, Belongia *et al.* (1997) found 8% and 15% white-tailed deer positive to granulocytic *Ehrlichia* by serology and PCR respectively. 16S rDNA sequence showed that the *Ehrlichia* was nearly identical to the published sequences for the agent of HGE, *E. phagocytophila* and *E. equi*. The results in our study suggest

a high prevalence of exposure/infection to *E. phagocytophila* in roe deer. The distribution of the pathogen appears to be very patchy and associated with higher number of ticks in the respective area. The high prevalence of PCR positive deer blood samples suggest that the animals were recently exposed to the pathogen since it is not detectable by PCR in experimentally infected sheep on day 14 in serum or day 17 in blood after inoculation (see Section 3.2). It is possible that under field conditions the animals are frequently reinfected with the pathogen due to continuous exposure to ticks.

Antibodies to *E. phagocytophila* can persist for several weeks in sheep, less long in cattle (Hudson, 1950; Foggie, 1960). That explains the high percentage of seropositive animals in areas with high tick densities representing either primary exposure or challenge. At site 4, positive animals by IFA and PCR were also found suggesting that *E. phagocytophila* can be maintained in the deer population even with very low tick numbers.

Deer may be an incidental host for infection with *E. phagocytophila*. Little is known about the pathogenesis of the disease in wild ruminants although it is assumed to be very mild. The presence of the organism in the spleen of the sampled deer suggest that the pathogen may be stored and recirculate to the blood stream during stress situations or indicates sequestration of infected neutrophils for their destruction. The capacity of *E. phagocytophila* to proliferate in roe deer and to enter *Ixodes* ticks feeding on the animal is very difficult to prove with this deer species by experimental transmissions because they are difficult to obtain and keep in captivity. However, the circumstantial evidence found in this study for the involvement of roe deer in a natural endemic cycle of *E. phagocytophila* transmission is as follows: (i) high seroprevalence; (ii) high infection prevalence of blood and spleen; (iii) all stages of the vector *I. ricinus* feed simultaneously on deer permitting transmission between tick instars during short patent bacteremias; (iv) *Ixodes ricinus* ticks were found infected with *E. phagocytophila* at a site where roe deer were also seropositive and where there were no sheep as alternative proven reservoirs; (v) and finally at a site with a sparse population of *I. ricinus* the seroprevalence and infection prevalence was significantly lower than at a similar site with a dense tick population (see Chapter

Four). Earlier experiments using roe deer blood induced TBF in sheep and cattle (MacDiarmid, 1965). Samples were obtained from an area where no sheep were present suggesting that domestic hosts are unnecessary for the survival of the pathogen.

Increasing numbers of deer in commercial conifer plantations may influence the epidemiology of TBF on adjacent sheep farms. However, there appears to be no reason for regarding roe deer infected with *E. phagocytophila* as a threat to sheep farming. In a recent study, the presence of deer next to farms in rural areas of England did not appear to increase the burden of ticks in the area or the prevalence of exposure to tick-borne diseases in humans (Thomas *et al.,* 1998). The management of TBF in endemic areas is based on ensuring exposure of lambs to natural infection from ticks to achieve endemic stability in the flock. Lambs are susceptible and acquire effective immunity whilst being naturally resistant to overt clinical disease. *Ixodes ricinus* in large areas of sheep farming land have a patchy distribution, which will make the managerial maintenance of endemic stability more difficult. Increasing deer and associated tick populations may decrease the patchiness of this distribution and thus tend to aid the maintenance of endemic stability. This hypothesis needs further specific field study to test it.

Two or more separate cycles of infection with *E. phagocytophila* are likely to occur in nature. The disease can be maintained in woodland habitats by deer, and probably rodents, in the absence of sheep or other domestic vertebrates. Recent research in Switzerland has identified 2.8% red foxes (*Vulpes vulpes*) positive by IFA for *E. phagocytophila* (Pusterla *et al.,* 1999a) suggesting many species of wild animals are exposed to the bacteria and thus confirming the wide range of hosts able to sustain granulocytic *Ehrlichia* infections. On the other hand, sheep alone can perpetuate disease cycles in UK uplands where they support all three feeding stages of tick (Ogden *et al.,* 1997).

Little is known about the prevalence of infection with *E. phagocytophila* or closely related granulocytic *Ehrlichia* in humans in the UK. The risk of tick borne *Ehrlichia* to humans in some countries is well established and additionally some authors suggested that granulocytic *Ehrlichia* can be transmitted to humans by

contact with infected deer blood (Bakken *et al.*, 1996). However, unrecognised tick bites may also be associated with those cases. With an infection prevalence up to 5% of nymphs of *I. ricinus*, human exposure to *E. phagocytophila* in these environments in the United Kingdom probably have numbered hundreds per year for many decades, and exposure is known to occur (Sumption *et al.*, 1995; Thomas *et al.*, 1998). There is no evidence of clinical disease of humans with the form of *E. phagocytophila* found in the United Kingdom. *Ixodes ricinus* nymphs have been detected to be co-infected with *B. burgdorferi* and 'HGE' by PCR in the UK (Guy *et al.*, 1998) suggesting there is a risk of concurrent infection after a single tick bite. However, primers for the study were not specific for HGE, therefore *E. phagocytophila* DNA was probably the pathogen amplified. There appears to be no reason to raise alarm but instead advocate continued vigilance for any change in the epidemiology of *E. phagocytophila* infection in the United Kingdom. Roe deer may serve as sentinels to identify areas where transmission of granulocytic *Ehrlichia* to humans and domestic animals may occur.

3.1.4.4 Abortion outbreaks associated with *E. phagocytophila* infection

Ehrlichia phagocytophila infection has been associated with abortion outbreaks in sheep and cattle. Naive pregnant animals usually abort when moved from free to tick-infested pastures (Jones and Davies, 1995). In this study, infection with *E. phagocytophila* was confirmed after detection of antibodies by IFA in aborted ewes and dams and amplification of the pathogens DNA in blood samples by PCR. Although not all the animals positive by IFA were positive by PCR, a combination of both techniques proved to be useful to detect an *E. phagocytophila* infection in the field. However, PCR does not seem to be sensitive enough to detect persistent infections in blood or sera (Stuen *et al.*, 1998). IFA is known to produce false positive results and cross-reactions with other *Ehrlichia* species are common. Thus PCR can be used to confirm the presence of the organism in the field for prevention and for epidemiological investigation purposes. In addition, PCR is a useful means of early diagnosis for this rickettsial pathogen, which is very difficult to grow in vitro. Thanks to this technique, minute quantities of bacteria present in the clinical sample

can by detected. A combination of PCR amplification and sequencing will provide a definitive identification of the organism. Antibodies to the bacteria can be retrospectively detected in convalescent serum samples.

PCR did not detect the bacteria in sera beyond day 14 in most of the infected animals as shown in a different experiment (Section 3.2). The pathogen was detected by PCR in blood as far as day 17 post experimental inoculation. Those findings suggest that although the organism is still present in blood it has lost its ability to infect granulocytes since elementary bodies cannot be readily detected free in sera. One of the animals was negative by IFA but positive by PCR indicating that the animal was in an early stage of infection when an antibody response was not developed yet. The rest of the flock, however, had cleared the organism from the sera but had already mounted an immune response. A different experiment (Section 3.2) showed that the bacteria could be detected in blood as early as day 5 after experimental inoculation when the antibody response starts to develop (Paxton and Scott, 1989).

Foetal fluid obtained from one of the aborted sheep was negative by IFA and PCR suggesting that vertical transmission does not occur although it has been suggested for cattle (Pusterla *et al.*, 1997a). Unfortunately, data from the foetal sample could not be linked with the maternal serum results.

3.2 Latent infection and tick transmission of E. phagocytophila *in sheep*

3.2.1 Introduction

It is believed that sheep become carriers after infection with *E. phagocytophila*. The bacteria can be detected after splenectomy 48 months after the infection has been cleared from peripheral blood (Stuen, 1993; Stuen *et al.,* 1998). The term 'carrier' is defined as a long term and low level of infection that is transmissible to ticks (or other vector) and thus to other mammal hosts as appears to be the case for *Theileria parva* and *Rhipicephalus appendiculatus* ticks (Kariuki *et al.,* 1995). Early experiments (MacLeod, 1936) determined that carrier state with *E. phagocytophila* occurred in sheep by subinoculation of blood and reproduction of the disease in susceptible animals when no parasites were observed in blood smears. *Ixodes* ticks that fed on infected animals were also able to transmit the disease to naive sheep but the experiment was carried out only during the acute phase of infection. It is not known if there is transmission of *E. phagocytophila* when there is an intensity of infection typical of the carrier state of the disease. It is possible that sheep only undergo latent infection with the bacteria thus, although the organism persists, the animals do not show clinical signs and the pathogen is not transmissible. Chronic infection with granulocytic *Ehrlichia* appears to occur in dogs (Egenvall *et al.,* 1997) with the animals showing mild signs of disease and persistent titres to *E. equi*.

Identification of carrier hosts is critical for management of clinically normal but persistently infected animals, which may represent a continuous source of rickettsia for transmission to susceptible animals. The aims of this study were to examine transmission rates in ticks and to determine if there was any significant difference in the infection of *Ixodes ricinus* fed as larvae on sheep with patent bacteremia with *E. phagocytophila* and on the same sheep when the infection had declined to less than 1% neutrophils showing morulae.

3.2.2.1 Tick transmission of *E. phagocytophila* to sheep

Field collected *I. ricinus* nymphs were allowed to feed to engorgement on sheep 369 (female Black-face) to assess infection in ticks. The sheep was monitored for the onset of the disease by daily measurement of its rectal temperature and observation of clinical signs.

3.2.2.2 Experimental inoculation of lambs with *E. phagocytophila*

Four sheep (Black-face) were obtained from a farm with no history of TBF and handled as follows. Their health was assessed prior to inoculation to detect any inadvertent infection that could interfere with the immune state of the sheep and with the transmission experiment. A blood sample of 10 ml in EDTA was taken and a blood smear was Giemsa stained and examined to detect any intraneutrophilic morulae. The rest of the blood was tested by PCR and IFA, using plasma obtained after centrifugation at 1000 *g* for 15 min, to confirm the animals were not previously exposed to *E. phagocytophila*.

All four experimental sheep were intravenously inoculated with 1 ml of a 1/10 dilution in PBS of *E. phagocytophila* Ehr/8 blood stabilate isolated in the Cree Wood in the South West of Scotland (see Chapter Seven). Sheep were monitored for the onset and course of infection by daily temperature measurement, at the same time every day to avoid diurnal variations, and observation of clinical signs. From day 3 blood smears from peripheral (ear puncture) and venous blood were Giemsa stained and examined by light microscopy (100 x lens). At least 100 neutrophils were counted and the intensity of infection (number of neutrophils showing *E. phagocytophila* characteristic intracytoplasmic morulae) expressed as a percentage.

Five ml of whole blood in EDTA K3 (B-P®) sterile vacutainers was collected for PCR purposes by jugular venipuncture from day 5 on alternate days to detect the parasite in blood during patent bacteremia and during the carrier state. Whole blood was also collected in sterile vacutainers with no additives (B-P®), allowed to clot incubated at 37°C during at least an hour, then placed at 4°C overnight. The obtained

sera was stored in Eppendorf tubes at -20°C until tested. Sera was taken on day 0 and weekly afterwards for IFA and PCR. IFA titres were compared from sheep during carrier state and patent bacteremia. On day 28 sheep were challenged with 1ml (1/10 dilution in PBS) of the same stabilate Ehr/8.

Two uninfected sheep were readily available for testing infection of the ticks feeding on the four experimentally inoculated lambs.

3.2.2.3 Experimental infection of *Ixodes ricinus* ticks and sheep

Uninfected larvae IXRI11, the product of 10 egg batches from adult female ticks that fed on sheep 1980 in May 96 and December 96, were available to feed on our four experimentally inoculated lambs (Fig 3.8).

Infected nymphs IXRI19 were derived from larvae fed on sheep 370 during patent bacteremia (when 27% of its neutrophils were found infected) in June 96. This sheep was experimentally inoculated with *E. phagocytophila* Ehr/8 blood stabilate derived from sheep 1980 that was infected by feeding field ticks collected from the wood of Cree. Infected nymphs IXRI19 were allowed to feed on sheep 369 in January 97 providing material for assessing methods of detection of infection in ticks (Section 3.2.2.1).

At day 2 of feeding half of the nymphs were removed from the sheep leaving the rest of the ticks for full engorgement. With half of the removed ticks haemolymph smears were stained with pararosaniline to detect *E. phagocytophila* in haemocyte cells. The remains of each tick were preserved in ethanol for use in PCR. Salivary glands of the batch of ticks that fully engorged were dissected and stained with Feulgen to detect masses of *E. phagocytophila*. The remains of the tick were also preserved for PCR. The prevalence (% of infected ticks) and intensity of infection (numbers of *E phagocytophila* in each infected tick) were measured.

Sheep 1419 was inoculated with Ehr/8 blood stabilate (derived from ticks in Cree wood). *Ixodes ricinus* nymphs IXRI35 were fed on sheep during their patent bacteremia. After sheep recovery (one month later) *I. ricinus* nymphs IXRI38 were fed on them.

Four lambs were experimentally inoculated with 1ml of 1/10 dilution in sterile PBS of Ehr/8 stabilate. From days 3 to 5 after experimental inoculation the four lambs were infested with 1000 uninfected larvae each of IXRI11 on the left ear, using an ear bag (Fig 3.9). Laboratory reared larvae were allowed to feed until detachment. Engorged larvae were cleaned and stored at 18°C, 100 % relative humidity, 18h of light for moulting and diapause. On day 18 after inoculation when less than 1% of the neutrophils were seen infected in Giemsa stained blood smears, a different batch of uninfected 1000 larvae were applied to the right ear of each sheep into ear bags. They were collected when detached, then cleaned and stored as before.

Moulted nymphs from the first and second feeding on sheep with patent bacteremia and carrier state were fed on uninfected sheep to assess tick ability to transmit the infection onto naive hosts.

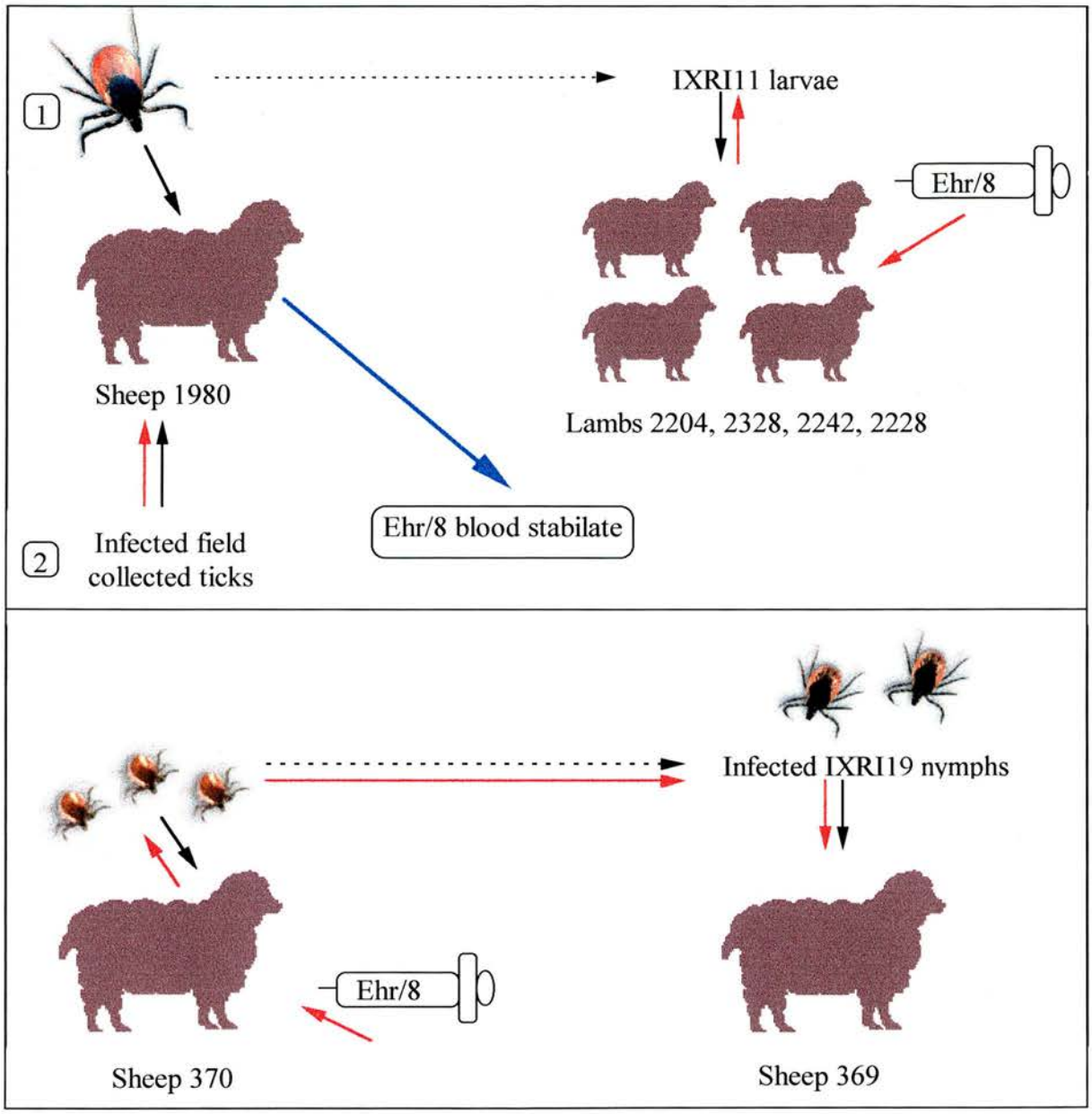


Fig 3.8 Summary of sheep and tick main experimental infections. Sheep 1980 was first used to maintain a tick colony then to isolate the *E. phagocytophila* Ehr/8 strain

→ Tick feeding

→ *Ehrlichia* infection (via syringe inoculation or tick feeding)

⇢ Tick moulting

Fig 3.9 *Ixodes ricinus* larvae were placed inside nylon bags glued to shaved and cleaned sheep ears and collected after full engorgement and detachment

3.2.2.4 DNA extraction from tick and sheep samples

DNA extraction from sheep blood in EDTA was performed using a QIAamp Tissue Kit (QIAGEN Ltd) kit following manufacturers' protocols as outlined in Chapter Seven in batches of four from the four sheep from the same day to avoid cross-contamination.

DNA from sera was extracted as follows. 250 µl of sera were aliquoted in 1.5 ml Eppendorf tubes. They were centrifuged at 18000 *g* for 15 min. The supernatant was discarded and 25 µl of lysis buffer containing 1 mM EDTA, 10 mM Tris-HCl, and 1% Tween 20 plus 10 µg of proteinase K (final concentration 400 ng/µl), were added to the pellet. The solution was heated to 55°C for an hour, then boiled for 10 min and stored at 4°C until use for PCR. 5 µl of the sample were used directly in PCR reactions.

50 salivary glands were dissected from field collected *Ixodes ricinus* nymphs and stored at -70°C in PBS until use. Genomic DNA was extracted from the salivary glands and 8 tick stabilates using a QIAamp Tissue Kit (QIAGEN Ltd). Nested PCR followed with primers and conditions described on section 3.2.2.5.

3.2.2.5 PCR in sheep blood and sera to amplify a fragment of 151-bp from the 16S rRNA gene

Single PCR with primers GER3-GER4 was used for the detection of *Ehrlichia phagocytophila* DNA in sheep blood and sera. They are known to be specific for the *E. phagocytophila* genogroup as previously described (Munderloh *et al.*, 1996b). Nested PCR was used for sheep samples to detect the bacteria during carrier state when less than 1% of the neutrophils were infected and persistently infected animals can not be detected by microscopy. For the outer amplification primers 856 and 1154 were designed according to the DNA sequences for the 16S rRNA gene for *E. phagocytophila* deposited in GenBank database (Fig 3.10). The primers were synthesised by Cruachem (Glasgow) with the following sequences:

856 5' GAATTGACGGGGACCCGCACAAGCG 3' and

1154 5' AAGGGCCGTGCTGACTTGACATCATC 3' (Appendix 3.1)

HGE has a gap in nucleotide position 886 (Chen *et al.*, 1994; Goodman *et al.*, 1996). Primer 856 contained a variation site that differentiates HGE isolate from *E. phagocytophila-E. equi*.

The PCR mixture contained for each reaction 500μM $MgCl_2$, 200μM of each dNTP (Boehringer Manheim), 0.2μM of each primer (Cruachem), 1.25 U of Taq DNA polymerase (Thermometric Ltd.), plus 5 μl of 10x reaction buffer containing 20mM Tris-HCl, pH 8.55, 16mM $(NH_4)_2SO_4$, 2.5 mM, 150 μg/ml of bovine serum albumin and 5 μl of DNA template in a final volume of 50 μl layered by 50 μl of sterile mineral oil (Sigma).

PCR conditions were the same for both PCR rounds except that the annealing temperature was higher (60°C) in the second round of amplification by using primers GER3-GER4. An initial cycle of denaturation at 95°C for 5 min was followed by 94°C for 1min, 50°C for 1 min, and 72°C for 1 min for 40 cycles, with a final extension step at 72°C for 7 min.

Agarose gels (2%) were stained with ethidium bromide (0.3 ng/μl). The agarose was dissolved in Loening-E buffer pH 7.6-7.8, containing Tris-base, NaH_2PO_4, and EDTA (Appendix C). Ten μl of the PCR products were mixed with 2μl of blue dye containing 75mM EDTA, 0.1% bromophenol blue, and 25% glycerol and loaded in the agarose gel. Samples were electrophoresed at 90 Volts for 45 min. The results were visualised using a UV transilluminator.

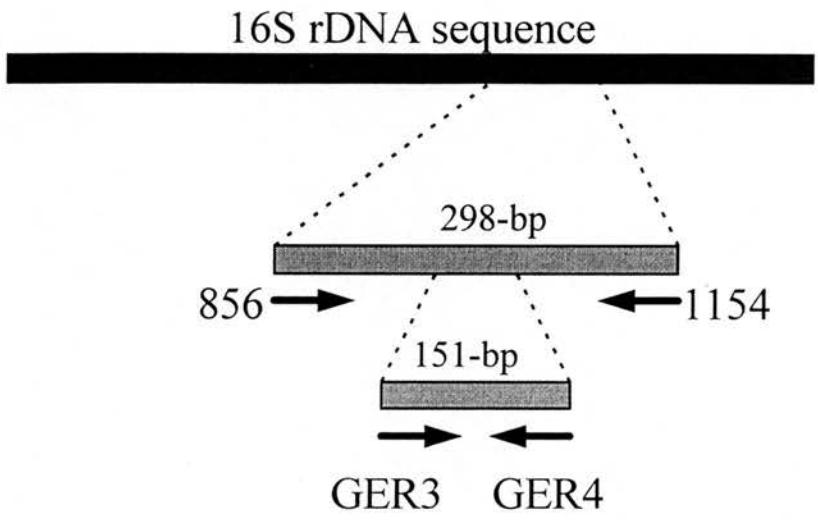


Fig 3.10 16S rRNA gene partial sequence. Location of primers (arrows) and size in base pairs of the PCR products

3.2.3 Results

3.2.3.1 Characteristics of experimentally induced Tick-borne fever in sheep

Sheep 369 showed signs typical of TBF from day 3 after tick exposure. Its temperature was high and Giemsa staining of blood smears showed the characteristic morulae inside the neutrophils from days 4 to 9. PCR was negative on days 2 and 3 but positive on days 6, 7, 9 during patent bacteremia and on day 10 when less than 1% of the neutrophils appeared infected in blood smears (Fig 3.11).

Infection was attained in all four lambs after experimental inoculation with Ehr/8 blood stabilate. On day 3 the rectal temperatures were high and persisted high until no bacteria were detected on blood smears. Then they fluctuated but no bacterium was seen with the exception of sheep 2228 that showed 2% bacteremia on day 17 when its temperature also rose to 41.7°C (Fig 3.12). Sheep showed characteristic clinical signs of *E. phagocytophila* infection such as dullness and lack of appetite. Their noses were wet and some of them showed a cough and mild respiratory distress. One sheep had a persistent high temperature and cough that resolved by itself. None of the animals needed treatment. By day 10 bacteremia was low and their clinical signs were inapparent.

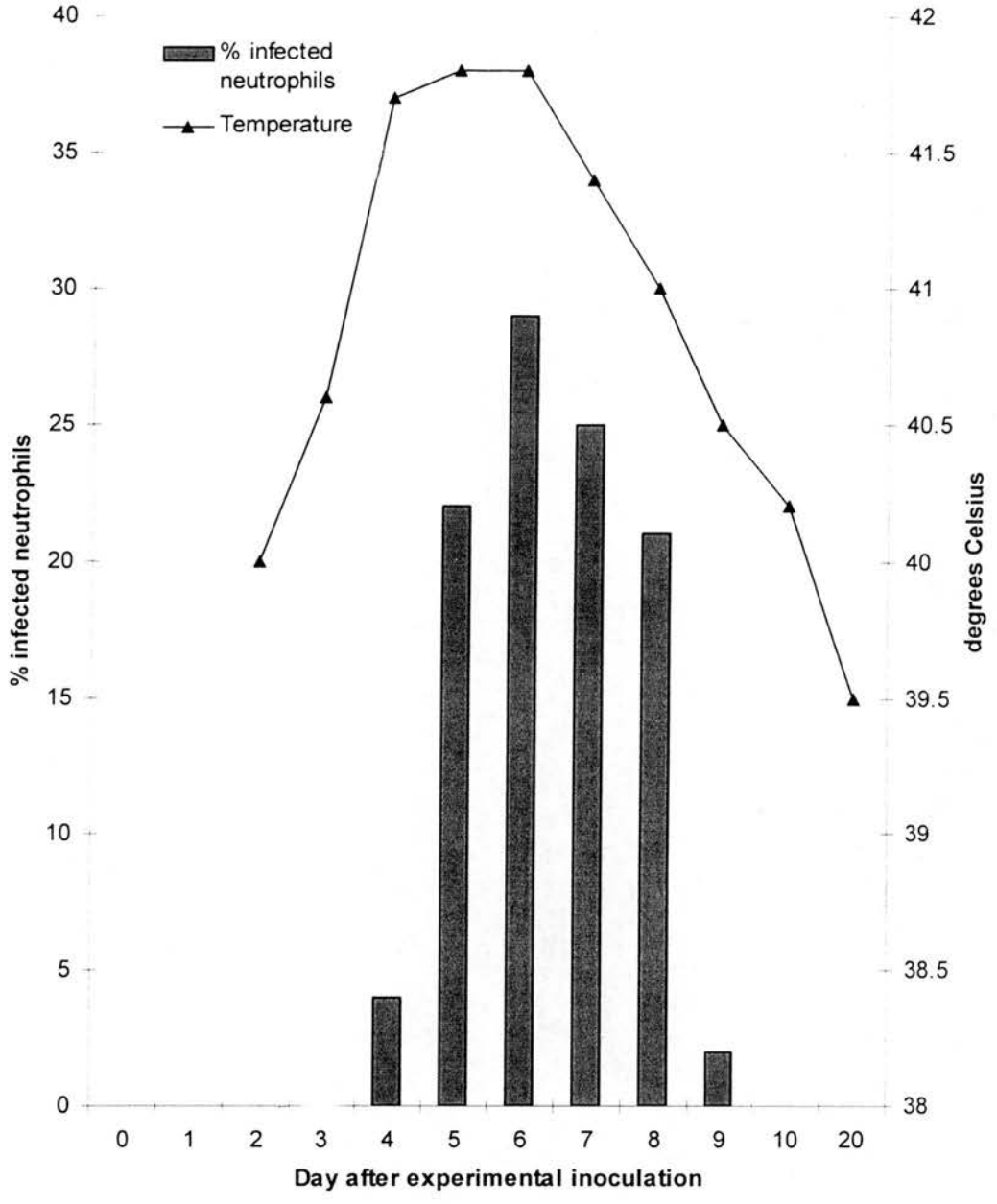


Fig 3.11 Temperature and percentage of infected neutrophils of sheep 369 after experimental infection using infected *I. ricinus* nymphs (IXRI 19). Sheep temperature was high from day 3 after exposure to ticks. Neutrophils were found infected in blood smears from days 4 to 9. PCR was negative on days 2 and 3 but positive on days 6, 7, 9 and 10

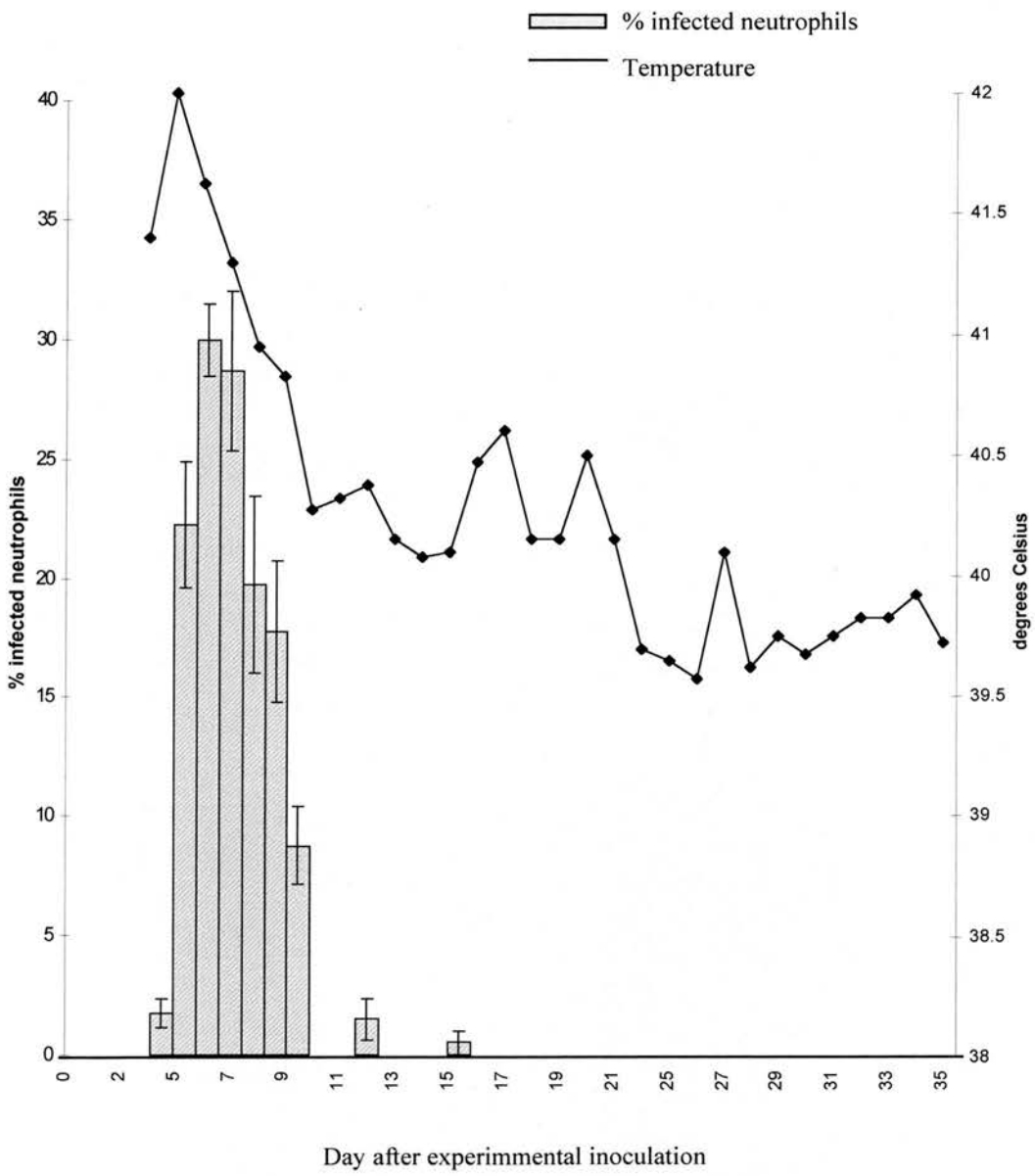


Fig 3.12 Mean temperatures and percentage of infected neutrophils in four lambs after experimental inoculation with Ehr/8 blood stabilate. Ticks fed on sheep from days 4 to 6 during patent bacteremia and days 18 to 20 during carrier state. Temperatures were high from day 3 and returned to normal from day 20. Standard errors of the mean for the temperature are included in Appendix 3.2.

3.2.3.2 PCR and IFA results after experimental inoculation of four lambs with *E. phagocytophila*

On day 0 prior to experimental inoculation all sheep were seronegative for *E. phagocytophila* as determined by IFA (Table 3.13). On day 7 titres were already high (>1/2560) in concordance with previous data (Paxton and Scott, 1989). Titres persisted high and raised to 1/10240 on day 21.

On day 0 none of the samples was positive by PCR. Positive results were obtained when using DNA prepared from sera and whole blood with specific primers GER3-GER4 from days 7 and 5 respectively. Sera and blood samples continued to be positive by PCR until days 14 and 17 respectively. All PCR products showed the expected size of 151-bp (Fig 3.13).

Table 3.13 Comparison of PCR, IFA and examination of Giemsa stained blood smear results after experimental inoculation of four lambs with *E. phagocytophila* Ehr/8 isolate

	PCR				IFA				Blood smears			
Sheep No.	2204	2328	2242	2228	2204	2328	2242	2228	2204	2328	2242	2228
0*	-	-	-	-	-	-	-	-	na	na	na	na
5	+	+	+	+	na	na	na	na	+	+	+	+
7	+	+	+	+	+	+	+	+	+	+	+	+
9	+	+	+	+	na	na	na	na	+	+	+	+
13	+	+	+	+	na	na	na	na	+	+	+	-
14	- (serum)	- (serum)	+ (serum)	- (serum)	+	+	+	+	na	na	na	na
17	+ (blood)	+ (blood)	- (blood)	+ (blood)	na	na	na	na	-	-	-	+
21	-	-	-	-	+	+	+	+	-	-	-	-
22	-	-	-	-	na	na	na	na	-	-	-	-
28	-	-	-	-	+	+	+	+	-	-	-	-
31	na	na	na	na	na	na	na	na	-	-	-	-
34	na	na	na	na	na	na	na	na	-	-	-	-
35	-	-	-	-	+	+	+	+	na	na	na	na

* Day after experimental inoculation
na Not available

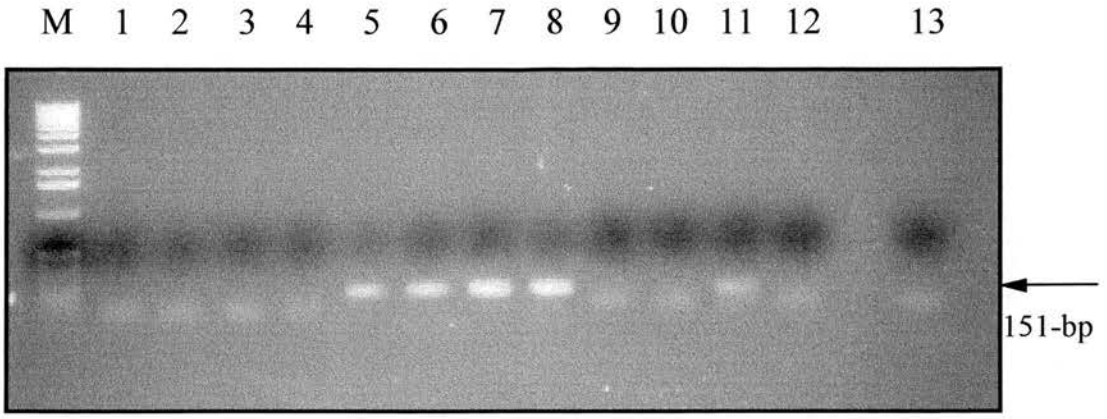


Fig 3.13 16S rDNA PCR using specific primers for granulocytic *Ehrlichia* to amplify 151-bp in serum samples from experimentally inoculated lambs with *E. phagocytophila*, Ehr/8 isolate. The 2% agarose gel was stained with ethidium bromide. A positive signal was obtained in all four lambs at day seven after experimental inoculation. Lamb 2242 was also positive at day 14. Lanes 1-4, serum from the four lambs, day 0; Lanes 5-8, day 7; Lanes 9-12, day 14; Lane 13, sterile distilled water

3.2.3.3 Tick feeding on experimentally infected lambs

Ixodes ricinus larvae successfully engorged during the patent bacteremia of experimentally inoculated lambs. Unfortunately most of them died during the processes of moulting thus they were unavailable for the second part of the experiment. It is not known why this occurred. Subsequent feeds on uninfected sheep to maintain the tick colony were also unsuccessful.

Second tick feeding on the right ear during carrier state was not as successful as the first one. Ears showed inflammatory pustulation and were hypersensitive to tick feeding. Many ticks were found dead during collection. Due to the difficulties in maintaining the colony of ticks alive the second part of the experiment, feeding nymphs that fed as larvae on sheep during carrier state and patent bacteremia on uninfected sheep, could not be pursued.

99 females and 88 males derived from IXRI35 and IXRI38 fed on sheep during patent bacteremia and after recovery were dissected. Feulgen staining of their salivary glands showed that none of the ticks were infected (Alan Walker, personal communication).

3.2.4 Discussion

There are very few studies to determine the efficiency of the carrier state host to infect ticks when *E. phagocytophila* cannot be detected in blood by laboratory means. An estimate of the coefficients of transmission between ticks and vertebrates is essential in order to understand the transmission dynamics of the disease in nature. The objectives of this study were to determine the influence of the intensity of infection in the mammal host on the transmission rate to ticks and to determine at what rate the pathogen passes from mammal host to ticks. The dynamics of transmission found in the study could be extrapolated to other tick-borne diseases. It was expected to be able to compare the infection rate on ticks feeding during patent bacteremia and carrier state and compare patterns of infection in non-infected sheep infested with ticks that pick up infection in both periods.

In general, threshold levels for tick infection with other tick-borne zoonoses are relatively high. *Ixodes trianguliceps* tick infection with *Babesia microti* requires at least 2% bacteremia in the main vector for the disease, the bank vole *Clethrionomys glareolus,* which only occurs during a brief period of five days (Randolph, 1992). It appears that infection of ticks is dose dependent, greater number of ticks becoming infected when higher number of pathogens are present in the vertebrate host (Nuttall *et al.,* 1992). High infection thresholds are also more efficient to infect *Rhipicephalus appendiculatus* ticks with *Theileria parva*, causative agent of East Coast Fever (ECF). However, carrier state, presumably associated with low *Theileria* infection levels in the vertebrate host, has been observed to be infective for ticks (Kariuki *et al.,* 1995). Unknown transmission mechanisms, probably involving tick saliva factors, remain to be discovered.

It is not known if sheep are responsible for the maintenance of *E. phagocytophila* infection in the new generation of ticks or if ticks are in fact responsible for the maintenance of infection in sheep or if it happens in both ways. Persistently infected sheep could be a continuous source of *E. phagocytophila* infection to ticks and susceptible sheep. It is not known if ticks gain infection better when feeding during patent bacteremia than during carrier state. It has been shown that sheep become resistant to tick infestation soon after the first feeding (Abdul-Amir and Gray, 1987). They develop immune responses that make it difficult for ticks to feed on the already exposed host. Only young lambs that join the herd each year are fully susceptible to successful tick feeding. Although more resistant to *Ehrlichia* infection than naive adults (Stuen, 1993) they undergo febrile symptoms and bacteremia. After patent bacteremia they might become carriers and be responsible for the maintenance of infection in the herd. Only nymphs and adults may be infected by *Ehrlichia* since transovarial transmission does not occur (MacLeod, 1936). In many *I. ricinus* habitats, ticks undergo two distinct peaks of activity during spring and autumn, with larvae and nymphs ticks questing at the same time in the vegetation (Gray, 1991). This pattern of activity corresponds with the presence of young hosts that are fully naive to both tick and *E. phagocytophila* infection, at least at the peak in April and May.

This study was on the ability of carrier state in sheep to maintain *E. phagocytophila* infection. Foggie (1951) detected the organism after months and sometimes even years when subinoculating blood from carrier animals into susceptible ones. Stuen (1993) detected the bacteria after splenectomy in blood smears 48 weeks after primary infection. Titres of IgM that can be detected over 12 months after *E. phagocytophila* infection and have been associated with carrier state (Woldehiwet and Scott, 1982a). There could be a low grade stimulation of the immune system by the antigen that results in the low-grade synthesis of IgM for prolonged periods. The bacteria is believed to persist circulating in blood, mesenteric glands, spleen, and central nervous system for prolonged periods in affected animals (Gordon *et al.*, 1932). However, *E. phagocytophila* does not persist in cattle blood for long periods (Hudson, 1950) and their immunity appears to be sterile. *Ehrlichia phagocytophila* experimental infection in sheep results in immunosuppression that lasts for at least 6 weeks (Larsen *et al.*, 1994). Not all antibody responses appeared impaired, infected sheep showed higher titres than the controls after exposure to *Actinomyces pyogenes* probably due to a higher exposure to the antigen because of the low number of circulating neutrophils.

The persistent antibody titres of both IgG and IgM to *Ehrlichia phagocytophila* suggest that a low persistent bacteremia triggers the immune reaction. *Ehrlichia* is an intracellular parasite, the antibody response is not the most efficient mechanism against the pathogen. Cell mediated immunity should play a more significant role. Elementary bodies may keep circulating in the blood stream at low levels, their proliferation controlled by the circulating antibodies, undetected in the blood smears but perhaps infective for ticks. Under stress situations, such as removal of the spleen, or possibly immunosuppression induced by ticks (Brossard and Wickel, 1997), the immune response is not able to control the parasite and they can invade neutrophils forming the characteristic morulae. Ticks can pick up infected neutrophils or even elementary bodies freely circulating in the sera. When *Ixodes* ticks feed for the first time on the host they induce a inflammatory accumulation of neutrophils in the site of tick bite (Brossard and Fivaz, 1982). Sensitised hosts mount a complex immune response involving localised, cell-mediated reaction at the site of

attachment, and humoral and complement-dependant mechanisms. In addition, factors such as host nutrition, sex and age may also contribute to the development of the immune response. Resistance to tick feeding has been described for the bank vole (*Clethrionomys glareolus*) (Dizij and Kurtenbach, 1995), a likely reservoir for granulocytic *Ehrlichia* spp. (Ogden *et al.*, 1998), characterised by an accumulation and degranulation of basophils (instead of neutrophils) at the site of tick attachment inducing an antibody and T-cell mediated release of tissue histamine and other substances increasing the vasopermeability, thus impairing tick feeding and inducing withdrawal or death (Brown, 1985). Even ticks that fully engorge may later on die after feeding on sensitised hosts producing a significant reduction in the moulting success (Sonenshine, 1993). Acquired resistance in vertebrate host seems to regulate tick burdens in nature and has an impact on pathogen transmission cycles. It appears to be immunomediated and is characterised by reduction in the number of attached and successfully fed ticks, impairment in the moulting success of fed immature ticks, and fecundity and fertility of fed female ticks.

Both humoral and cellular factors are involved in the acquired resistance to tick feeding. On the other hand, tick feeding induces immunosuppression in *Apodemus* and BALB/c mice species, the yield of ticks is increased after each infestation and enhanced feeding success is also observed (Dizij and Kurtenbach, 1995; Dusbabek *et al.*, 1995). No effect on T-cells occurs but B-cells appear suppressed and there is a reduction in the total quantity of generated antibodies. A reduction in the number of skin mast cells follows which contributes to the enhanced tick feeding. In addition, basophils are absent in mice and tick saliva enzymes can destroy vasoactive substances secreted by the rodent (Ribeiro and Spielman, 1986) thus ticks can feed repeatedly on them but are rejected by other common wild hosts such as voles (Davidar *et al.*, 1989). Wood mice are able to control *Borrelia* spirochetes (Kurtenbach *et al.*, 1994) and *Babesia microti* infections (Randolph, 1994) developing specific immune responses that correlated with low transmission rates to non-infected ticks thus they are poor hosts for the pathogens. It is likely that blood ingested by the tick contains both pathogens and humoral components from the vertebrate host thus inducing parasite destruction within the tick. *Babesia microti*

infection, although it has immunosuppressive effects, does not disrupt the development of acquired resistance to tick feeding in voles (Randolph, 1994). Furthermore, bank voles show little immunity to spirochetes and develop high levels of infection that increase the transmission rates to ticks in at least 3-fold when compared to ticks fed on wood mice (Kurtenbach *et al.*, 1994). This evidence suggests that vertebrate host immunity and infection threshold to *Borrelia* and *Babesia* are important regulatory factors in the horizontal transmission to the ticks. It has been observed that immune rejection is less common in long established host-tick relationships (in terms of geological time) as appears to occur with *I. dammini* and white-footed mice (Spielman *et al.*, 1985). The development and maintenance of tick-host specificity depends on tick secretion of immunosuppressant, anti-inflammatory, and antihaemostatic agents with the saliva targeted against the natural hosts (Ribeiro *et al.*, 1985) to suppress their rejection response. It is known that tick salivary gland proteins change during the feeding period (Wheeler *et al.*, 1989). Pathogens carried by ticks may have an effect in their own biology. Because large numbers of virus or bacteria in the blood meal are potentially dangerous for tick survival, it is possible that ticks secrete different salivary factors or in different concentration depending on the host to which they attach to induce resistance in those animals potentially infected with large numbers of organisms to reduce the load of pathogens included in the blood meal.

Ixodes nymphs failed to pick up infection when feeding on sheep during patent bacteremia and persistent infection. None of the dissected adults examined after moulting appeared infected with *E. phagocytophila*. This can be due to lack of sufficient ticks, particularly adults, feeding on sheep to induce immunosuppression such that *Ehrlichia* can be efficiently transmitted. Tick induced immunosuppression is known to enhance pathogen establishment within the vertebrate host (Wikel and Bergman, 1997). It is also possible that although the pathogen was transmissible to sheep, it was not well adapted to allow infection of the laboratory colony of ticks feeding on sheep. In Lyme borreliosis, natural transmission of the spirochetes via ticks induces higher transmission rates to the vector than experimental transmission via syringe (Shih and Liu, 1996). There is virulence variance between different *E.*

phagocytophila strains (Foggie, 1951; Foggie, 1960; Foster and Cameron, 1970a). It is not known if the use of this particular isolate (Ehr/8) would make a difference in the tick and sheep responses and/or if it could be extrapolated to the rest of isolates. Sequence divergence for the 16S rRNA gene at the strain level within the genus *Ehrlichia* is virtually non-existent. The sequences of Old Sourhope and Feral Goat strains are identical for that gene (Anderson *et al.*, 1991) although some degree of genetic diversity between European granulocytic isolates derived from cattle and sheep appears to occur (Engvall *et al.*, 1996; Ogden *et al.*, 1998). Further differences have been found at *groEL* gene level between different isolates of *E. phagocytophila* (Chapter Seven). It is believed that Ehr/8 isolate derived from ticks that fed on roe deer because this is the most abundant host of the area from where ticks were collected. Sequence analysis at *groEL* level revealed a close similarity of Ehr/8 to *E. phagocytophila* isolates (Chapter Seven) indicating that it belonged to the *E. phagocytophila* complex and therefore typical patterns of disease on sheep and infectivity for ticks would be expected when using this isolate. It is also possible that due to the young age and good state of health of the experimental animals they were able to effectively clear circulating *Ehrlichia* soon after inoculation, thus inhibiting tick infection.

Foggie (1951) suggested that both plasma and serum were infective, thus some pathogenic factors must be present outside neutrophils. In this study the presence of the organism was demonstrated in blood and sera on days 17 and 14 respectively thus showing that the bacteria can be found free in sera until at least day 14. PCR might not be sensitive enough to detect low levels of free elementary bodies persisting in the blood stream.

It was not possible to perform xenodiagnostic studies to determine the infectivity of the mammal host to vector ticks. Although ticks fed well on experimentally infected sheep during their patent bacteremia they did not survive the moulting process. Sheep are known to acquire resistance to *Ixodes ricinus* tick feeding (Abdul-Amir and Gray, 1987) although they used adults, which are less immunogenic than larvae. The reported immune response was similar to the observed in *Clethrionomys*, a neutrophil infiltrate followed by basophils that soon

degranulated, mononuclear cells, then lymphocytes dominated. Feeding *I. ricinus* larvae were very immunogenic as shown by the hypersensitivity observed on the ears after second tick feeding. Larvae are known to be the most immunogenic stage when compared to nymphs and adults (Uhlir *et al.*, 1994). Most of the larvae feeding during carrier state did not engorge and were collected dead. Death in situ of ticks feeding in already exposed sheep has been reported (Abdul-Amir and Gray, 1987) probably associated with a direct toxic effect of immune reaction mediators.

Despite the fact that sheep become persistently infected with *E. phagocytophila* the significance of sheep as a carrier in the epidemiology of the disease is still to be determined. They could be mainly a provider of the blood meal for female adult ticks necessary for egg laying and maintenance of tick populations. *Ixodes ricinus* ticks are indiscriminate feeders (Anderson, 1989). Larvae and nymphs can feed on small mammals, birds, and reptiles. Adult ticks feed mainly in medium-large sized wild and domestic mammals. However, all stages of tick can be found feeding simultaneously on large mammals such as roe deer as shown in Chapter Four. Sheep may also be a host for all stages of tick. However the routine use of acaricides on sheep populations may impair tick feeding, the number of larvae and nymphs that feed on sheep may not be enough to pick up the infection and maintain the disease under field conditions.

Clethrionomys and *Peromyscus* species of rodents appear to be competent reservoirs for *Borrelia burgdorferi* and *Babesia microti* in Europe and the United States (Piesman, 1988; Randolph, 1991, 1994). They are closely related species that differ from *Apodemus,* which is more related to mice. However because *Clethrionomys* acquires resistance to tick feeding it may reduce its reservoir potential in situations when there are high tick densities and reexposure is frequent.

Sheep or large mammals can act as providers of blood meals and also amplifiers of infection in ticks without the need of a disseminated infection. Little is known about the role of co-feeding (transmission of infection from infected to non-infected ticks which is not dependant on the development of viraemia by the vertebrate host thus non-viraemic sheep and many other mammals can serve as hosts) and amplification (an infected vertebrate host gives rise to more than one infected

tick vector) of *E. phagocytophila*. Co-feeding is a mechanism of pathogen amplification which appears to occur for tick-borne encephalitis virus (Labuda *et al.*, 1993), *Borrelia burgdorferi* (Gern and Rais, 1996) and louping-ill virus (Hudson *et al.*, 1995) through tick saliva factors when non-infected ticks feed next to infected ones. In the case of *B. burgdorferi* the persistence of the organism localised in the skin facilitates the transmission via co-feeding in rodents (Gern and Rais, 1996). Despite sheep and deer not developing systemic infections of *Borrelia* spirochetes capable of infecting larvae ticks (Telford *et al.*, 1988; Jaenson and Talleklint, 1992), localised non-systemic transmission to nymphs appears to occur in the case of sheep (Randolph *et al.*, 1996). Thus a wide range of vertebrate species that do not develop systemic infections may contribute to the transmission of tick-borne diseases. Low levels of infection with *E. phagocytophila* in sheep could be amplified thanks to co-feeding. That would help in the maintenance of *Ehrlichia* infections in nature. The mobility and function of neutrophils are known to be impaired by both *E. phagocytophila* infection and tick infestation (Foster and Cameron, 1970b; Woldehiwet and Scott, 1982d; Ribeiro *et al.*, 1990). *Ehrlichia phagocytophila* may be more prevalent in peripheral neutrophils on the capillaries where the organism would be readily available for ticks following systemic or non-systemic infection of the vertebrate host.

CHAPTER FOUR, EPIDEMIOLOGY OF GRANULOCYTIC *EHRLICHIA* PATHOGENS IN THE VECTOR TICK *IXODES RICINUS*

4.1 Infection prevalence with Ehrlichia phagocytophila *in field collected* Ixodes ricinus *nymphs*

4.1.1 Introduction

Ehrlichia spp. are tick-borne pathogens of humans and animals. There is an increased interest on the study of these organisms due to the recent diagnosis of a granulocytic *Ehrlichia*, genetically closely related to the ruminant causative agent of TBF, affecting humans, dogs, and horses in Europe (Johansson *et al.*, 1995; Clark *et al.*, 1996), and the US (Chen *et al.*, 1994; Greig *et al.*, 1996). It is believed that the recently identified granulocytic isolates could be strain variations of *E. phagocytophila*. Although the vector for the newly diagnosed human granulocytic ehrlichiosis has not been confirmed, it is suspected to belong to the genus *Ixodes* (Pancholi *et al.*, 1995). Vector competence has been demonstrated for a strain of HGE using *I. dammini* ticks in a laboratory rodent model (Telford *et al.*, 1996). Granulocytic *Ehrlichia* has been also detected by PCR in *Ixodes trianguliceps* ticks of the UK (Ogden *et al.*, 1998). However, these ticks are a nidicolous species, which means they only feed on burrowing small mammals such as rodents and therefore it is unlikely they will transmit any infection to large mammals and humans, although they may maintain enzootic cycles of zoonotic diseases (Randolph, 1995).

Ehrlichia (Cytoecetes) phagocytophila is transmitted by *Ixodes ricinus* ticks (MacLeod and Gordon, 1933). Only nymphs and adult ticks are considered to be vectors for *E. phagocytophila* since transovarial transmission was found not to occur (MacLeod, 1936). However, methods for detection of infection in ticks may not be sensitive enough. Newly developed molecular techniques will allow for the detection of minute quantities of DNA and the screening of large number of samples for the presence of pathogens in a short period of time.

The aim of this study was to determine the natural prevalence of infection in vector *Ixodes ricinus* ticks by using various methods to confirm the results. Ticks for the study came from presumed sites of natural infection in deer adjacent to known foci of TBF in sheep (Fig 4.1 and 4.2). Ticks were collected predominantly from vegetation because this precludes the complication with feeding ticks which may

contain infected fresh blood. Woodland sites inhabited by deer were predominantly sampled for ticks because of the much higher abundance of ticks there in comparison to sheep pastures.

A knowledge of the transmission rate would assist a better control of TBF in sheep by managerial maintenance of endemic stability. This depends on allowing sufficient exposure of lambs to infected ticks when they are relatively resistant to clinical TBF but able to acquire immunity (Stuen, 1993). However, ticks are not only vectors for disease, they can also induce anemia, toxicosis, weight loss and hide damage thus emphasising the need for a control in the numbers of ticks that attach to domestic and wild hosts.

The infection prevalence in ticks is an essential characteristic in the overall transmission rate to sheep but little is known about the prevalence of *E. phagocytophila* in field ticks. The existing estimates of prevalence of infection in ticks are very high (MacLeod, 1936; Webster and Mitchell, 1989). It leads to the assumption that exposure to any individual *I. ricinus* tick will infect lambs (Henderson, 1990). Those figures need corroboration with different and newly developed methods.

Fig 4.1 Typical habitat for *Ixodes ricinus* and *E. phagocytophila*. Cattle and sheep grazing together adjacent to woodland inhabited by deer

Fig 4.2 Satellite image showing the location of collection sites for *I. ricinus* ticks in Scotland (Red arrow). 1, Edinburgh; 2, Glasgow

4.1.2 Materials and Methods

4.1.2.1 Field collection of ticks

Questing ticks were collected from vegetation by cloth dragging in standardised transects on 20 occasions during the period March to September in 1996 and 1997 (Fig 4.3 and 4.4). The cloth drags were 1x1 m, of towelling made of white cotton in loop stitch. They had a bar in sleeves at each end and a loop of cord for pulling at one end. The transects consisted of 10 m drags, separated by at least 10 m, each drag taking approximately 25 seconds. They were made in fixed lines on each sampling occasion. All ticks were removed with forceps and stored in universal tubes with damp paper for return live to the laboratory (Fig 4.4). Preserved sub-samples were identified using the key in Hillyard (1996). Four sites were used, Nos. 1 to 3 in South West Scotland and No. 4 in South East Scotland. Each site was characterised as sheep farms on upland grazing bordering onto woodland. At each site roe deer (*Capreolus capreolus*) were present in the woods and at Site 1 there were in addition red deer (*Cervus elaphus*). The deer also occasionally used the sheep pasture for grazing but sheep were restricted to the pasture by fences except for rare entry into the woods. Samples were collected from both types of wood, the sheep pastures, and on one occasion directly from sheep at Site 4.

To obtain an estimate of the prevalence of *E. phagocytophila* infection in the population of host-seeking ticks that were feeding on roe deer sites, in September 1998 nymphs and adults were collected from vegetation by cloth dragging along grassed rides from two conifer forests (sites 4 and 5, see Chapter Three) on two occasions at each site and during similar mild and dry weather conditions. In total 40 drags were made for site 4 and 45 for site 5. Tick salivary glands were examined for infection with *E. phagocytophila* using methods of Alberdi *et al.* (1998) as summarised in sections 4.1.2.3 and 4.1.2.4.

Fig 4.3 Two scenes illustrating tick collection by blanket dragging in pasture vegetation from Cree, located in the South of Scotland

Fig 4.4 Tick collection by blanket dragging in Cree vegetation adjacent to a coniferous wood. Ticks attached to the cloth were removed using forceps and stored in universal tubes until arrival to the laboratory

4.1.2.2 Tick counts from roe deer legs

Immediately after arrival roe deer legs (see Chapter Three) were stored at -20°C. Ticks were washed from the containing plastic bag and filtered in a sieve of 125 μm pore diameter. Each leg was allowed to thaw and the hair separated with the help of forceps to sample for ticks remaining attached on the legs. For each leg five searching transects were made along its length, aided by a stereoscopic microscope. A small number of ears (8) of roe deer paired to legs were similarly examined. Ticks collected from roe deer were not examined for infection with *E. phagocytophila* because many of them were in some state of engorgement and the sole presence of the bacteria within the vector only indicates that the pathogen was contained in the blood meal.

4.1.2.3 Salivary gland dissection

Salivary glands of nymphs and adults were removed by dissection from the whole live ticks when stuck to wax and immersed in saline. The guts were separated from the ticks then the glands were removed and placed in drop of 1% serum albumin in 1% saline on a microscope slide. In the case of nymphs the guts were removed then the entire mass of viscera were placed in one operation onto the slide. The liquid drops were spread out and the glands teased out in the film of liquid. The slides were dried then fixed for 1h in 10% formalin. The glands were stained by the Feulgen method (Bancroft and Stevens, 1982), which is specific for DNA. One batch of 50 ticks had been fed for 2 days on a rabbit to stimulate maturation of the *Ehrlichia*; the rest were from unfed ticks and without any other treatment.

4.1.2.4 Tick salivary glands staining by Feulgen and IFAT

Indirect fluorescent antibody test (IFAT) was used as previously described (Paxton and Scott, 1989) with some modifications. Dissected paired tick salivary glands were used as antigen. Microscope slides containing the salivary glands were fixed for 15 min in acetone and air dried. A well was made by circling the salivary glands with a ball-point paint marker (Texpen BDH). Tick material appears to be naturally fluorescent. To reduce non-specific fluorescence antisera dilutions ranged

from 1/50 to 1/200, the FITC conjugate dilutions ranged from 1/80 to 1/160 in PBS, PBS containing 1% Tween 20, PBS containing 1% normal rabbit serum, or PBS containing 1% Evans blue counterstain (Sigma). Best results were obtained with the following protocol. Non-specific reactions were blocked by incubating with normal rabbit serum at a 1/20 dilution in PBS (Phosphate buffered saline, pH 8.0), for 30 min. The slides were then washed three times in PBS and sheep antiserum of high anti-*Ehrlichia phagocytophila* titre (1/10000) was added at a dilution of 1/100 in PBS containing 1% rabbit serum. Each pair of salivary glands was flooded with 50µl of the diluted sera and incubated in humid chamber for 1h. The slides were washed three times in PBS for a total of 15 min and 50µl of an anti-sheep fluorescein isothiocyanate anti-IgG (whole molecule) conjugate (Sigma) raised in donkey was added at a dilution of 1/160 in PBS containing 1% rabbit serum. The slides were incubated for 45 min in a humid chamber. After 3 washes in PBS the slides were allowed to dry before mounting in phosphate-buffered glycerol (90%) for viewing under UV light.

After use for IFAT coverslips and glycerol were removed, and the salivary glands were stained with Feulgen to determine the morphology after Feulgen staining of the bodies considered to react with *E. phagocytophila* antiserum by IFAT. The staining was performed as previously described (Bancroft and Stevens, 1982) as follows. The slides were fixed in 10% formalin for 60 min. After a wash in water, hydrolysis followed in 1N HCl at 60°C for 10 min. Then the slides were placed directly into Schiff's reagent (Appendix A) at 20°C. They were kept in dark to stain for 60 min at 4°C then washed in potassium metabisulphite 10% solution for 2 min. After a wash in tap water, the slides were allowed to dry then mounted with DPX plastic mountant and a coverslip.

4.1.2.5 DNA extraction from *I. ricinus* ticks

Field collected *Ixodes ricinus* nymphs were partially fed on rabbit ears to stimulate the pathogen activation (Piesman and Spielman, 1982) and collected with forceps on day 2 of feeding. They were cleaned with warm water and placed in 1.5 ml Eppendorf tubes in batches of 10. Tick batches were overlaid with 50 µl of buffer

TE containing 10 mM Tris, 1 mM EDTA, and 1% Tween 20, then ground with the help of plastic tips until the external chitinous material was eliminated from the tick. Then 10 µg of proteinase K (final concentration 200 ng/µl) were added. The resulting sample was incubated at 55°C for at least three hours, then boiled for 10 min to inactivate proteinase K. The samples were further processed following methods described by Barlough *et al.* (1996) with some modifications. Tick samples were centrifuged at 13000 *g* for 15 min to pellet the insoluble chitinous material and the supernatant transferred to fresh sterile 1.5 ml Eppendorf tubes. Three volumes of absolute ice cold ethanol plus 100 µl of 3 M sodium acetate were added to the supernatant and the mixture was kept at -20°C to precipitate the DNA. After 48 h the samples were centrifuged at 18000 *g* for 15 min to pellet the DNA. The supernatant was removed and the pellet washed twice in 70% ice-cold ethanol, then resuspended in 100 µl of sterile distilled water and stored at -20°C until use in PCR.

DNA from tick stabilates and a batch of 50 dissected nymph *Ixodes ricinus* salivary glands was extracted using a QIAamp Tissue Kit (QIAGEN Ltd.) following manufacturers' instructions. The protocol was the same as described in Chapter Seven for blood samples with slight modifications. Prior to incubation at 70°C for 10 min the samples were incubated in buffer ATL, containing proteinase K (final concentration 2 mg/ml), at 55°C for 3 h.

4.1.2.6 Nested PCR using primers to amplify 151-bp fragment of *E. phagocytophila* 16S rRNA gene from *I. ricinus* ticks

Sixteen batches of 10 ticks each from two different sites, a pool of 50 tick salivary glands, and 8 tick stabilates were tested by nested PCR to assess or confirm their prevalence of infection. PCR reaction mixture and conditions were the same as described in Chapter Three to amplify a fragment of 151-bp from the 16S rDNA sequence of *E. phagocytophila.*

4.1.2.7 PCR amplification to detect *E. phagocytophila* DNA in *I. ricinus* larvae using 16S rDNA and *groE* genes specific primers

Ixodes ricinus larvae were collected from the vegetation by cloth dragging as previously described. 1200 ticks were divided in batches of 100 and their DNA extracted following methods outlined in 4.2.2.2. PCR for the 16S rRNA and *groE* genes followed, by primers and conditions previously described in Chapter Three. As a positive control, DNA extracted by a QIAamp Blood Kit (QIAGEN Ltd.) from a heparinised blood sample from a parasitaemic sheep was used.

4.1.3 Results

4.1.3.1 Field collection of ticks

No ticks other than *Ixodes ricinus* were found in the sub-samples and it is assumed that all ticks examined were this species. Collaborative studies by A. R. Walker from this Department showed a bimodal pattern of questing activity of ticks, particularly the nymphs (Fig 4.5; Table 4.1). The great majority of ticks were found in the woodlands and during the April-May peak of activity, but there was a secondary peak in September. Tick numbers on sheep pasture, for equivalent sampling effort, were so low that only 20 nymphs were tested for infection.

Ticks from vegetation on deer site 6 comprised 135 nymphs and 38 adults of *I. ricinus*. Only one female *I. ricinus* was found at site 4 (see tick numbers from both sites in Appendix 4.2).

Table 4.1 Mean and standard deviation on the numbers of different instars of *Ixodes ricinus* ticks collected in Scotland throughout the year

		larvae		nymphs		adults	
month	n	mean	SD	mean	SD	mean	SD
Jan	3	0	0	0.24	0.051	0.179	0.147
Feb	3	0	0	0.961	0.295	0.302	0.136
Mar	6	0.04	0.095	1.162	0.329	0.343	0.243
Apr	6	0.919	0.718	1.608	0.332	0.406	0.137
May	7	1.395	0.53	1.581	0.26	0.457	0.234
Jun	7	1.055	0.665	1.098	0.329	0.234	0.108
Jul	6	0.525	0.712	1.071	0.28	0.245	0.09
Aug	6	0.757	0.859	0.719	0.234	0.161	0.094
Sep	5	0.969	0.433	0.934	0.334	0.249	0.099
Oct	5	0.095	0.141	0.579	0.326	0.132	0.081
Nov	3	0.032	0.045	0.505	0.322	0.24	0.226
Dec	3	0	0	0.302	0.104	0.117	0.083

n number of sampling occasions per month

mean logarithmic means of ticks ($\Sigma(\log_{10} n+1)/n$)

SD standard deviation from the mean

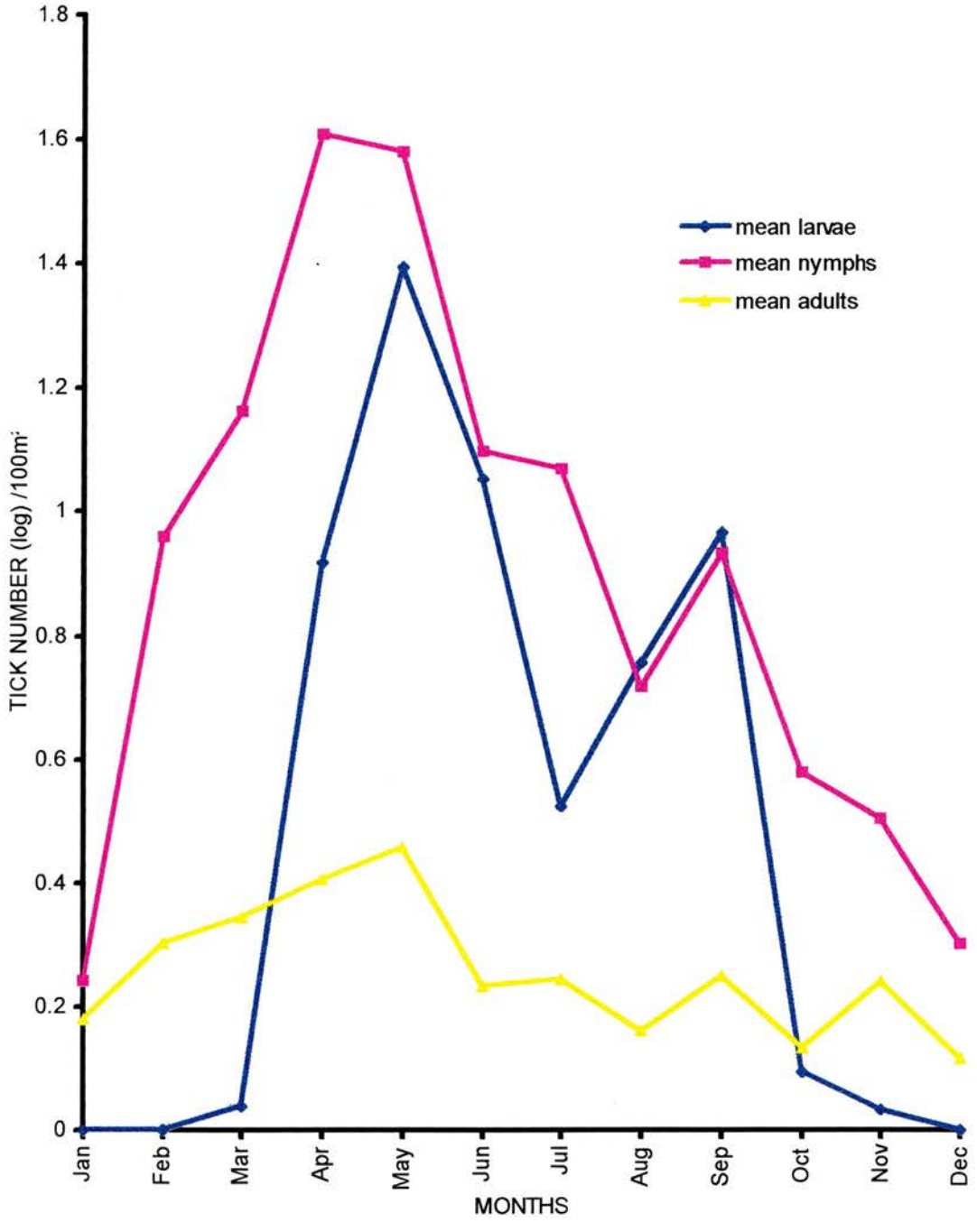


Fig 4.5 *Ixodes ricinus* - Seasonal fluctuations of questing activity in Scotland (data kindly provided by Dr. Alan R. Walker)

4.1.3.2 Roe deer legs tick counts

Of the 71 roe deer legs examined 59 (83%) had tick infestations, exclusively of *Ixodes ricinus*. All instars of *I. ricinus* simultaneously parasitised deer, particularly during the main activity period in spring. Nymphs were found from March through to November. There was wide variation in infestation levels between the sites, as shown in Table 4.2, which corresponded to the higher prevalences of exposure and infection with *E. phagocytophila* in roe deer of the same sites. Eight roe deer ears were similarly examined and the number of ticks found compared to the results in paired legs (Table 4.3). On average, less larvae and nymphs were found on ears but a few more adults. Thus in general, sampling of roe deer legs is a better indicator of tick infestation levels than ears. A positive correlation was found in the numbers of larvae and nymphs present on the same individual hosts (Fig 4.6) as observed in previous studies (Craine *et al.*, 1995). The mean density of parasites per host (total number of ticks divided by the total number of hosts) was 49 and the intensity of infestation (number of ticks divided by the number of infested hosts) was 56.

Table 4.2 Numbers of different instars of *Ixodes ricinus* ticks (larvae, nymphs and adults) observed on roe deer legs from nine widespread sites in the UK. Mean numbers of ticks and range for each site are shown

		Mean numbers of ticks (range)					
Site	No. legs	Larvae		Nymphs		Adults (male)	
1	1	29		6		0	
2	14	11	(0-57)	4	(0-18)	0.07	(0-1)
3	10	26	(2-48)	14	(3-40)	0.13	(0-1)
4	2	2	(0-4)	0		0	
5	8	0.8	(0-5)	0		0	
6	2	21	(1-48)	3	(0-8)	0	
7	15	63	(4-454)	25	(4-99)	0.07	(0-1)
8	18	74	(0-636)	10	(2-34)	0	
9	1	1*		0		0	
Total	71	2671		727		3	

* Not *I. ricinus*

Table 4.3 Mean number of ticks found on eight roe deer ears paired to ticks found on legs

	Mean numbers of ticks	
Instar of tick	Legs	Ears
Larvae	93.9	6.6
Nymphs	75.6	29.3
Adults	0.1	2.1

$\chi^2_{df=1} = 13.88$, Yates corrected, $p<0.01$

The difference in the numbers of ticks found on roe deer legs compared to the ears from the same animal was statistically significant. Only larvae and nymphs were considered, the numbers of adults were too small to be compared. Overall, legs give a better estimate of the abundance of ticks observed on deer although the distribution of different instars of ticks on several parts of the hosts varies.

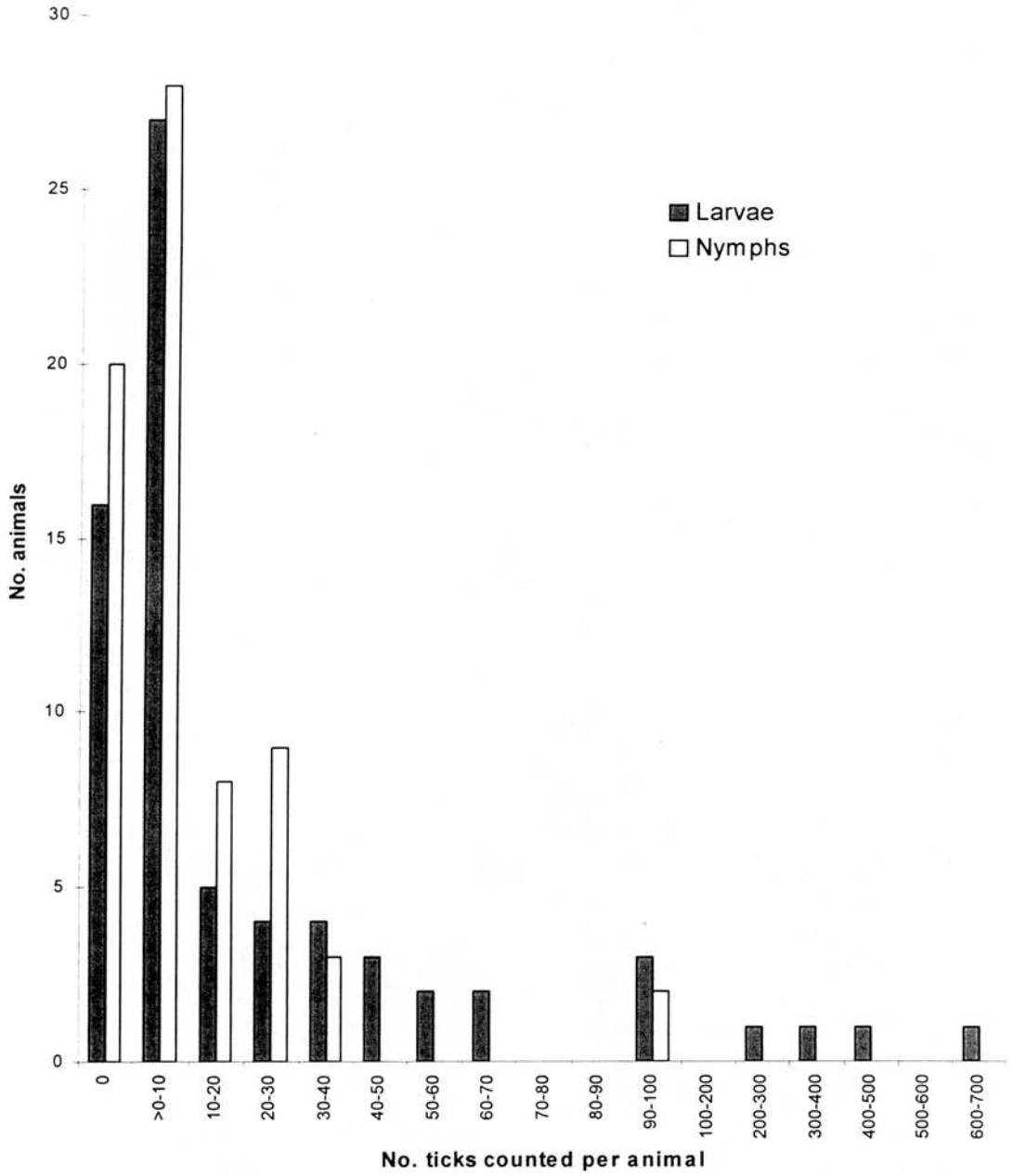


Fig 4.6 Frequency distribution of larvae and nymphs found on individual roe deer legs during March 97-October 98. Only three animals had adult tick infestations (1 tick each). Most animals carried few ticks and a few deer carried large numbers of larvae and nymphs

4.1.3.3 Assessment of infection with *E. phagocytophila* in dissected tick salivary glands after staining with Feulgen and IFAT

A total of 554 ticks were dissected and their salivary glands examined by Feulgen staining with the collaboration of A. R. Walker. Typical preparations are shown in Figure 4.7. The overall infection prevalences shown by Feulgen staining of salivary glands was 1.4% in nymphs, 1.6% in females, and 2.1% in males (Table 4.4). All positive ticks were collected from woodland. Table 4.5 shows the intensity of infection of the ticks, in most of the ticks considered infected the salivary glands were dominated by the masses of presumed *Ehrlichia*, with most salivary acini infected with one or more masses. Intensity was defined as the number of individuals of a parasite in each infected host. Type 1 acini were not seen infected. In the unfed ticks the masses were large relative to the host cell and stained densely and conspicuously. In the one positive salivary gland (Fig. 4.7.b) from the batch fed for 2 days on a rabbit, many of the *Ehrlichia* masses were substantially enlarged relative to those seen in unfed nymphs (Fig. 4.7.a) but no hypertrophy of the host cell nucleus was seen.

Of the batch of 111 nymph salivary glands tested by IFAT one was positive (Fig. 4.8.b), and the positive masses had similar morphology to presumed *Ehrlichia* stained with Feulgen. When these same glands were subsequently stained by Feulgen none of the remaining 81 was positive. The other 30 pairs of glands were lost during processing of the IFAT preparations, including the preparation positive by IFAT. A negative gland is compared to a positive gland in figure 4.8.

135 nymphs and 38 adult ticks were collected from vegetation on roe deer sites. They were also examined for salivary gland infection by Feulgen staining. None of the adults but 7 nymphs (5%) were positive. It correlated to the high number of ticks found on deer and to the high percentage of PCR and IFA positive deer from this same site (Tables 4.2, 3.10, and 3.12).

Table 4.4 Summary of the prevalence of infection in field collected *I. ricinus* ticks according to IFA and Feulgen staining

	IFA		Feulgen	
Type of tick	No. positive/No. tested	%	No. positive/No. tested	%
Nymph	1/111	0.9	6/445	1.4
Female	-	-	1/61	1.6
Male	-	-	1/48	2.1

- No ticks were tested

$\chi^2_{df=2} = 0.18$, $p>0.05$

Not statistically significant

Table 4.5 Numbers of *Ehrlichia* masses per infected tick observed in Feulgen stained salivary glands (Intensity of infection)

	No. positive	No. masses
Nymphs	6	19, 64, 88, 90, 130, 140
Female	1	384
Male	1	20

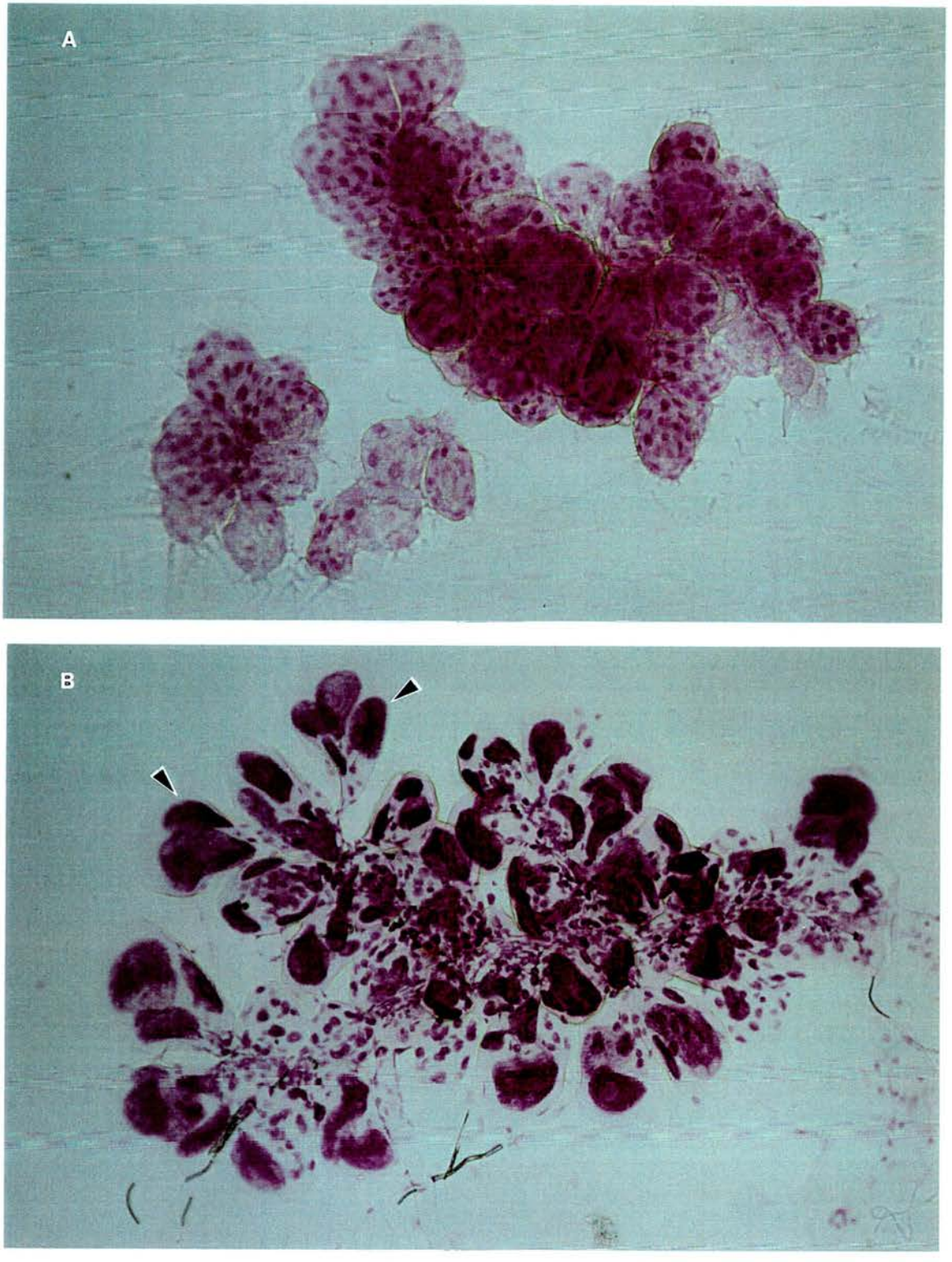


Fig 4.7 Dissected salivary glands from nymph *I. ricinus* ticks (A) Negative by Feulgen staining (B) Positive by Feulgen staining (arrowheads) (x220)

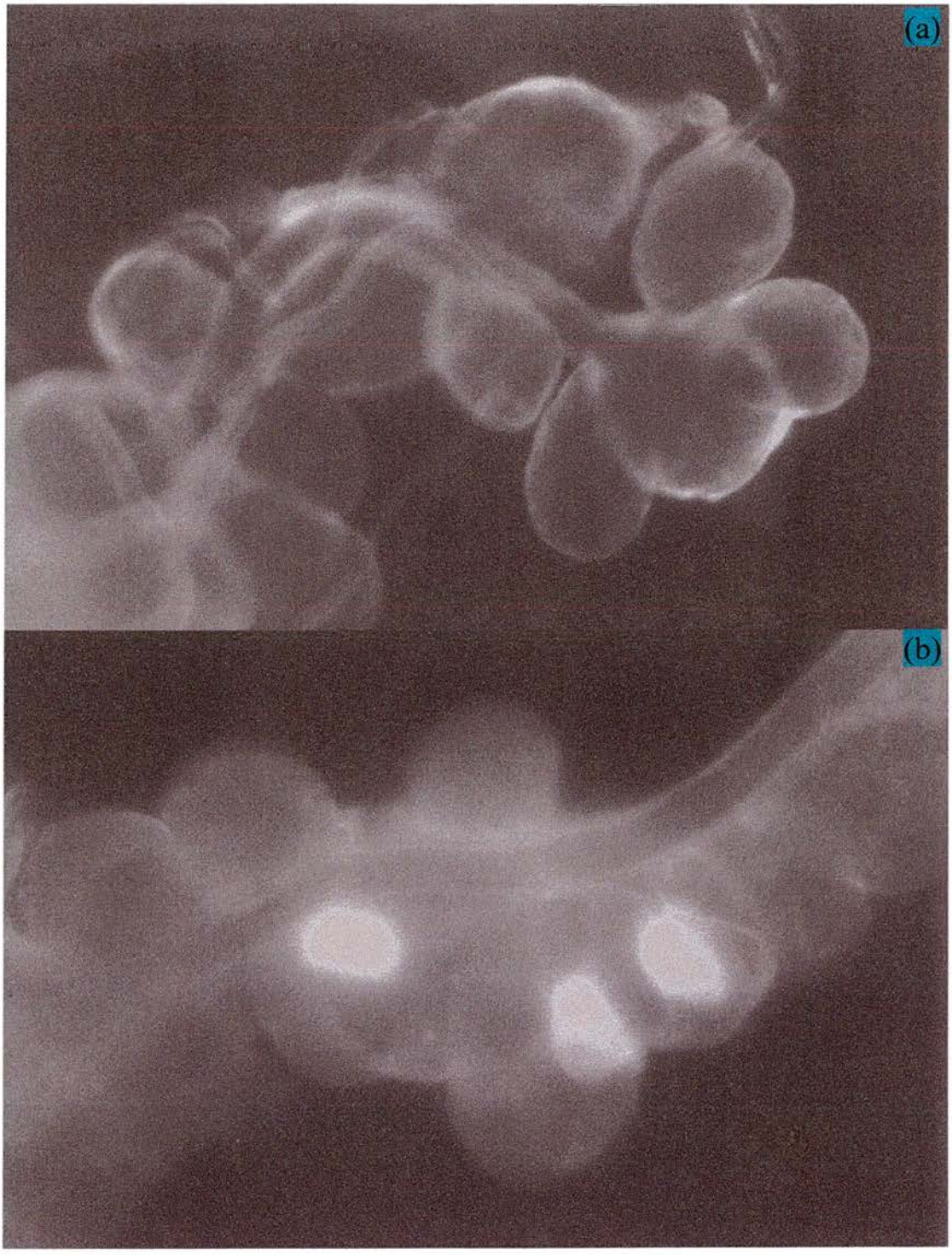


Fig 4.8 Dissected salivary glands from nymph *I. ricinus* ticks (a) Negative by IFA (b) Positive by IFA staining (x480)

4.1.3.4 *Ehrlichia phagocytophila* DNA amplification from ticks after nested PCR (16S gene)

The prevalence of infection in ticks was calculated using the equation given by Chiang and Reeves (1962), which has been also used to determine the prevalence of *Cowdria* infection in *Amblyomma* ticks by Norval *et al.* (1990).

$$p = 1-((n-X)/n)^{1/m}$$

p prevalence

n number of pools (batches) tested

m mean number of ticks per pool

X number of pools positive

Infection rates were calculated (Table 4.6) for the 16 batches of 10 fed ticks, one batch of 50 salivary glands from nymphs which had been pre-fed for two days, 12 batches of 100 field collected larvae and 8 batches of 50 nymphs fed for 2 days and prepared as stabilate for inoculation into sheep tested by nested PCR and reamplification of a 5 µl aliquot of the PCR product using again the internal pair of primers (GER3-GER4, Munderloh *et al.*, 1996b).

Table 4.6 Estimated infection rates with *E. phagocytophila* in *I. ricinus* ticks after PCR testing

	m	n	X	p
Batches of 10 fed ticks	10	16	1	0.64%
Salivary glands	50	1	1	≥ 2% *
Tick stabilates (nested PCR)	50	8	1	0.27%
Tick stabilates (re-amplification)	50	8	8	≥ 2% *
Batches of 100 larvae	100	12	0	0

* When all pools were positive the lowest rate possible was based on a single tick of each pool being positive

4.1.3.5 Assessment of transovarial transmission after PCR amplification of *E. phagocytophila* DNA using 16S rRNA and *groE* genes specific primers

Only DNA from the positive control containing *E. phagocytophila* was amplified after PCR. None of the twelve batches of larvae appeared positive (Fig 4.9) indicating that transovarial transmission does not occur or at a very low rate, with a prevalence of infection in newly hatched larvae of less than 1/1200 (Table 4.6).

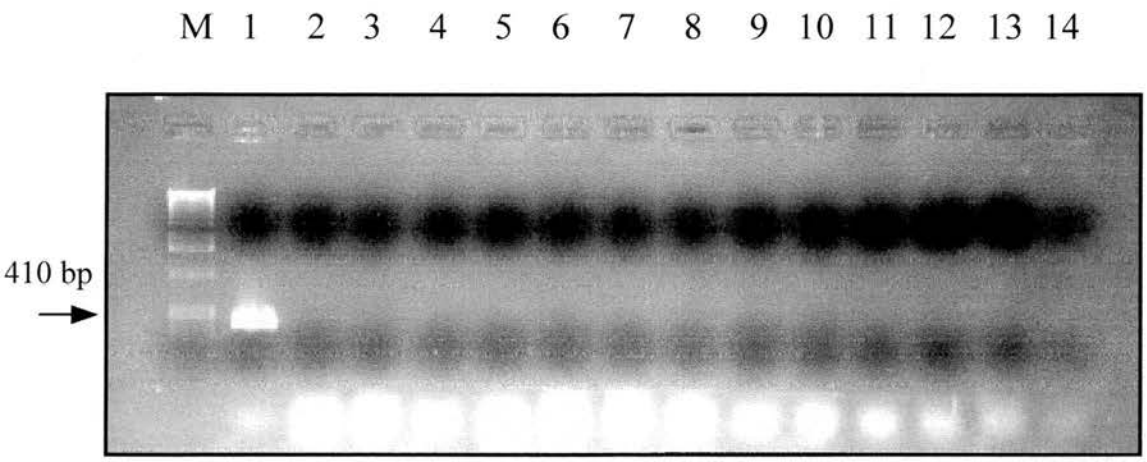


Fig 4.9 *GroEL* PCR using specific primers for granulocytic *Ehrlichia* to amplify 410-bp (arrow) from 12 batches of *I. ricinus* ticks containing 100 larvae each. The 1% agarose gel was stained with ethidium bromide. M, molecular size marker, 1Kb; Lane 1, blood from sheep experimentally inoculated with *E. phagocytophila*; lanes 2 to 13, batches of *I. ricinus* larvae; lane 14, sterile distilled water

4.1.4 Discussion

A low prevalence of infection was detected in the studied ticks. The results are consistent in all our tests suggesting a truly low prevalence of infection. Our findings are in agreement with the infection rates detected in ticks from California (Barlough *et al.*, 1996) but not with the data from the eastern and Midwestern United States (Magnarelli *et al.*, 1995b) or previous research in Scotland (Webster and Mitchell, 1989). Ticks for this current study were collected largely in woodland sites and very probably relate to foci of natural infection in deer, whereas in the latter study, ticks were infected by feeding in experimental sheep, and tested by the method of antibody coated electron microscopy grids. Recent research performed in Switzerland (Pusterla *et al.*, 1999b) has detected also very low prevalences of infection with *E. phagocytophila* by PCR in all stages of field collected *Ixodes ricinus*. Several factors such as regional or seasonal variability of *E. phagocytophila*-infected ticks or *Ehrlichia* life cycle may have an effect on the prevalence of *E. phagocytophila* in ticks.

Of the various methods of assessing infection in ticks Feulgen staining was the simplest and it also gave data on intensity of infection. It has the great advantage of indicating infection that is likely to be competent for transmission at the next feed because the *Ehrlichia* has developed in cells of the salivary glands. However it is not specific for *Ehrlichia* and dissection of unfed nymphs requires manual skill. Feulgen staining will also detect *Babesia* sporoblasts in *Ixodes* salivary glands (Petrov, 1948) but these sporoblasts are very large and additionally induce massive hypertrophy of the host cell nucleus. The IFAT can be used in conjunction with Feulgen staining in order to determine the identity of infection with granulocytic *Ehrlichia*. PCR was a specific technique that identified the tick batches as truly infected with *Ehrlichia phagocytophila*-like organisms. Due to the low infection prevalence this complex method is not ideal for routine epidemiological survey using individual ticks in these localities but should be used, in conjunction with Feulgen staining, to verify the identity of the *Ehrlichia* in batches of ticks. PCR may have sensitivity problems producing false negatives depending on DNA extraction procedures, or Taq enzyme inhibitors (Higgins and Azad, 1995). Misleading positives may be produced by PCR

used at high sensitivity because of the tendency for vector borne pathogens to enter many individual vectors in high numbers but then be killed by the defences of a high proportion of the vector population. Residual DNA from the killed pathogens or from live pathogen in a location in the vector unsuitable for transmission or in too small amount to be infective, may be detected by PCR when used to extremes of sensitivity. This may explain the discrepancy between our results with the 8 batches of 50 ticks tested by inoculation into sheep, and by low sensitivity and high sensitivity PCR.

Information on the control of TBF in sheep often implies that all *Ixodes ricinus* on sheep pastures in endemic areas are likely to be infected (Henderson, 1990). Our data are not consistent with this but tick populations supported by sheep may have higher prevalences of infection as observed in different studies (Ogden *et al.*, 1998) and this needs to be investigated further.

Adult ticks are expected to have higher prevalences of infection because larvae and nymphs may maintain infection even after feeding on refractory hosts (Webster and Mitchell, 1989). Our results from small samples of adults do not corroborate this finding. Field infestation of deer and sheep with nymphs and adults at time of peak tick activity (April-May) at these sites would exceed tens of nymphs attaching per day, and at least 1 female per day (Milne, 1948), thus exposure of every animal to *E. phagocytophila* remains likely.

Nymph and adult *Ixodes ricinus* can survive for at least one year (MacLeod, 1932), thus they are capable of being long term reservoirs of infection. Mechanisms in transmission such as co-feeding have not been reported in natural hosts for this organism. Early transmission experiments performed by MacLeod (1936) concluded that the carrier state was infective to ticks and therefore it would be expected that tick infection rate should be high, but our results are not compatible with this. It is of great epidemiological importance to verify the role of carrier infections in sheep and deer in the maintenance of infection. Similar estimations of infection prevalence in field ticks are also needed for risk assessment of diseases of potential public health importance in this study area, such as Lyme borreliosis, human granulocytic ehrlichiosis, and babesiosis.

Site 6 showed a higher percentage of infected ticks (5%) than the 1% to 2% in our previous studies of other sites (Alberdi *et al.*, 1998). Ogden *et al.* (1998) found also low but variable prevalences depending on woodland or upland locations suggesting a wide variation in the prevalence of infection in ticks depending on the area. Interestingly, significantly higher prevalences of infection were observed in upland ticks, which corresponded with all three feeding stages of tick feeding mainly upon sheep. Low prevalences of infection in ticks have been also found in *Rhipicephalus appendiculatus* with the protozoon *Theileria parva*, causative agent of East Coast Fever (Walker and Fletcher, 1984). It appears that the pathogen requires a high infection threshold within the vertebrate host because the majority of organisms ingested with the blood meal are destroyed in the midgut. However, despite only 10% of ticks achieving high levels of parasitaemia the disease can be spread if ticks become abundant (Young *et al.*, 1992). Another rickettsia, *Cowdria ruminantium*, also appears to have varied rates of infection in ticks ranging from <1% to 45% (Norval *et al.*, 1990).

The presence of granulocytic *Ehrlichia* in ticks of separate areas of Britain where alternative mammal reservoir hosts are found indicates that endemic cycles of infection can be maintained in both domestic sheep and wild animals. Adult *Ixodes trianguliceps* ticks appeared to contain granulocytic *Ehrlichia* after PCR analysis (Ogden *et al.*, 1998). However, engorged ticks collected from rodents were studied. The sole presence of the pathogen within the tick does not demonstrate vector competence, it may only indicate that the bacteria was included in the blood meal. In contrast, it does not prove reservoir status for the rodents because adult ticks could have acquired the bacteria from a previous meal. In our study, ticks collected from shot roe deer were not analysed for infection with *E. phagocytophila* because most of the ticks were at some stage of engorgement, thus a positive result could be attributable either to the presence of the bacteria in deer or the tick, and would be therefore inconclusive.

Ticks have a skewed distribution on vertebrate hosts in nature, a few individuals are exposed to heavy infestations due to the clustered questing of larvae that derive from the same batch of eggs (Labuda *et al.*, 1993). This was also observed

on this study when counting numbers of ticks found on roe deer legs. There was a positive correlation between large numbers of larvae and nymphs found in a few hosts and small numbers of ticks found in most of the roe deer legs. Numbers of ticks found on a leg is an underestimation of the real abundance of ticks infesting deer. Nevertheless, leg sampling proved to be a useful and less laborious method than the collection of the whole hide from the carcass which can also give an underestimation because many ticks may manage to escape during the process.

In a previous experiment (see Chapter three) indirect evidence to support a major role of roe deer as reservoir for *E. phagocytophila* in the UK was found. However, confirmation is needed by isolation of the bacteria from field samples in culture, which has not been attained hitherto. Another means for confirmation would be the collection of *I. ricinus* larvae from field deer to examine them for infection after moulting to nymphs. The obtained prevalence could be compared to the one determined from host seeking nymphs collected by blanket dragging in the same region.

4.2 Identification of the blood meal source for Ixodes ricinus *nymphs when they fed as larvae*

4.2.1 Introduction

The cytochrome b gene is a protein coding sequence, which has been widely used to determine phylogenetic relationship in mammals (Irwin *et al.*, 1991). The main advantage of the cytochrome b protein is that it is encoded by the mitochondrial genome that evolves in a different rate than the rest of the cell DNA. The cytochrome b is part of the 9-10 proteins that make up complex III of the mitochondrial oxidative phosphorylation system. It appears to be heterologous enough between vertebrate species to identify them at the genus level (Kirstein and Gray, 1996).

It is of epidemiological interest to determine the blood feeding hosts of *I. ricinus*. Those vertebrate hosts may be potential reservoirs for *E. phagocytophila* in nature, their identification will help to determine risk factors and control measures. Previous studies have pointed to deer and rodents as the main reservoirs for *Ehrlichia* pathogens (Telford *et al.*, 1996; Ogden *et al.*, 1998; Alberdi *et al.*, submitted for publication). Bank vole and wood mice have been identified as important host for larval *I. ricinus* in UK woodlands, but they seem to carry few nymphs and adults (Randolph and Craine, 1995; Randolph *et al.*, 1996). In the absence of transovarial transmission, the prevalence of granulocytic *Ehrlichia* infection in those hosts may be low since they receive few infective bites from adults and nymphs. In contrast, *Peromyscus leucopus* (white-footed mice) in the US appear to be competent reservoirs for granulocytic *Ehrlichia* (Telford *et al.*, 1996; Des Vignes and Fish, 1997) with a high infection prevalence probably associated with the large numbers of *I. scapularis* larvae and nymphs that feed on them (Levine *et al.*, 1985). Pheasants, which appear to be competent reservoirs for Lyme spirochetes, seem to be very inefficient for granulocytic *Ehrlichia* transmission to ticks (Ogden *et al.*, 1998) and they also carry fewer larvae than nymphs.

The aim of this study was to determine the source of the blood meal as larvae from field collected questing *Ixodes ricinus* nymphs in a woodland site in Scotland, endemic for *E. phagocytophila*. The following mammal hosts have been found at the

tick sampling site (A.R. Walker, personal communication): Roe deer, red deer, sheep, cattle, wood mouse, field vole, shrew, badgers, foxes, humans, dogs, weasel and red squirrel. Neither rabbits nor hares were found and the presence of bank vole was not confirmed.

4.2.2 Materials and Methods

4.2.2.1 DNA extraction from vertebrate hosts

Genomic DNA from the following mammal hosts: roe deer (*Capreolus capreolus*), fallow deer (*Dama dama*), sheep (*Ovis aries*), goat (*Capra hircus*), bank vole (*Clethrionomys glareolus*), wood mouse (*Apodemus sylvaticus*), cattle (*Bos taurus*), Balb/C mice (*Mus musculus*), rabbit (*Oryctolagus cuniculus*), rat (*Rattus rattus*) and shrew (unknown species) was extracted with the QIAamp Tissue Kit (QIAGEN Ltd) from blood, plasma or spleen following manufacturers' instructions as previously described in this chapter and in chapter seven. Genomic DNA from field vole (*Microtus agrestis*) was not available.

4.2.2.2 DNA extraction from individual *Ixodes ricinus* nymphs from pasture, deciduous, and coniferous forests in Cree

DNA was extracted from *I. ricinus* ticks collected from pasture, deciduous and coniferous areas of the forest of Cree by cloth dragging as previously described (Section 4.1.2.5) with some modifications. Individual nymphs were placed in 1.5 ml Eppendorf tubes and covered by 50 µl of buffer for tick digestion (10 mM Tris, 1mM EDTA, 1% Tween 20, 200 ng/µl proteinase K). Ticks were crushed with the tip of a pipette and disrupted by vigorous pipetting, then incubated at 55°C for at least 3 h and at 37°C overnight. Samples were boiled for 10 min to inactivate proteinase K and, after a brief centrifugation at 6000 *g* to pellet the insoluble chitinous material, the supernatant was used directly for PCR amplification.

4.2.2.3 PCR amplification of the host cytochrome b with degenerate primers

PCR by previously described degenerate primers (Kirstein and Gray, 1996) (Appendix 3.1) amplified mitochondrial sequences containing fragments of the cytochrome b from the host DNA. Primers cytb1-cytb3 were used to amplify a 638-bp fragment. To increase the sensitivity of the test, nested PCR was chosen for the 95-bp fragment. Primers 2cytb1-2 cytb6 were followed by primers 3cytb1-2cytb5 for a final 95-bp PCR product (Fig 4.10). PCR conditions were the same for both rounds as follows: Thirty cycles of denaturation at 94°C for 1 min, annealing at 55°C for 2 min and extension at 72°C for 1.5 min, followed by a final extension at 72°C for 5 min (Omnigene thermal cycler, Hybaid).

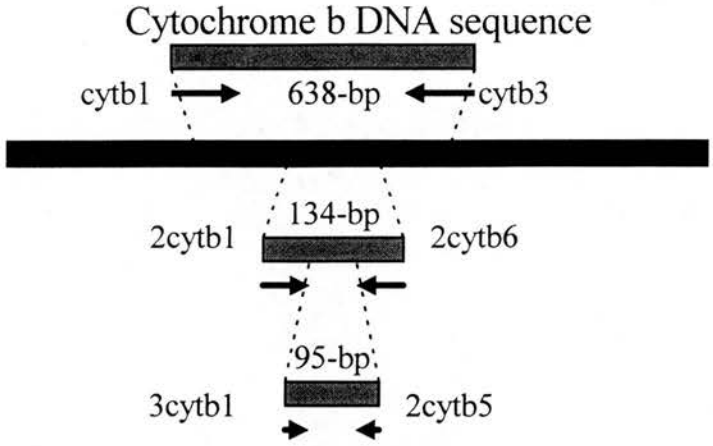


Fig 4.10 Cytochrome b partial sequence. Location and orientation of primers (arrows) and size in base pairs of the PCR products

4.2.2.4 Detection of PCR products by agarose gel electrophoresis

Amplified products were detected on 1% agarose gels (Sigma) in Tris Borate EDTA (TBE) buffer pH 8.0. Ethidium bromide (0.3 ng/µl) was added prior to the setting of the gel. Wide slot combs were used for the gels and 10 µl of reaction products mixed with 1.5 µl of loading buffer (7x concentrated) were loaded onto the gel. A 1kb DNA ladder was also included as a marker. Electrophoresis was performed in 1 x TBE buffer at 70 volts for 40 min. Products were then viewed using a UV transilluminator.

4.2.2.5 Enzymatic digestion of 638 and 95-bp PCR products from mammal hosts (RFLP, restriction fragment length polymorphism analysis)

Restriction enzymes HaeIII and DdeI were used to cut the 638-bp fragment, the 95-bp PCR product was digested with enzymes HaeIII and MboII. Those enzymes were chosen because they appeared to be useful to discriminate between several vertebrate species in previous studies (Kirstein and Gray, 1996).

4.2.2.6 Cloning and sequencing of a 638-bp fragment of the cytochrome b gene amplified from roe deer

A 638-bp fragment from the cytochrome b from roe deer was amplified by primers cytb1-cytb3 (Kirstein and Gray, 1996). PCR conditions were modified to allow 10 min of final extension at 72°C. Amplicons obtained by PCR from a roe deer spleen sample were cloned using TA Cloning® Kit (Invitrogen BV, The Netherlands), following manufacturers' protocols as described in Chapter Seven. Plasmid DNA was isolated using Wizard miniprep system (Promega, Madison, USA) according to manufacturers' instructions. The presence of the purified insert was confirmed by restriction digestion with EcoRI enzyme. Following cloning the isolate was sequenced in both directions by automated sequence analysis (I. Bennett, Department of Veterinary Pathology, Royal (Dick) School Of Veterinary Studies, University of Edinburgh).

4.2.2.7 Sequence analysis and design of an internal probe for roe deer based on the 638-bp cytochrome b DNA amplicon

The sequence obtained from roe deer was aligned with previously described sequences from sheep, cattle, mice (Bibb *et al.*, 1981), fallow deer and black-tailed deer (*Odocoileus hemionus*) (Irwin *et al.*, 1991). Although the whole 638-bp fragment sequence was available, the probe was designed based on the 95-bp that could be amplified from ticks. A mouse probe was used as described by Kirstein and Gray (1996) as a general probe for rodents in the hope that it would react to the variety of species present in the field. Both oligonucleotide probes were synthethised by MWG-Biotech (Germany). The probes were used at a concentration of 20 ng/ml.

4.2.2.8 Digoxigenin labelling of 95- bp DNA fragments obtained after PCR amplification

95-bp PCR products from sheep, roe deer, bank vole, wood mice and Balb/C mice were labelled using DIG DNA labelling and detection kit (Boehringer Mannheim) following manufacturers' protocols. By this method, labelled nucleic acid molecules are generated with Klenow enzyme polymerase by random primed incorporation of a digoxigenin-labelled deoxyuridinetriphosphate. Briefly, 2 µl of each PCR product were diluted with sterile distilled water to a final volume of 15 µl. DNA was denatured by boiling for 10 min then quickly chilled on ice. Two µl of denatured DNA, 2µl of hexanucleotide mixture (10x), 2µl of dNTP labelling mixture, 10 µl of dH_2O, and 1 µl of Klenow enzyme (final concentration 100 U/ml) were mixed together and incubated at 37°C overnight. Two µl of 0.2 M EDTA, pH 8, were added to stop the labelling reaction and the probes were stored at -20°C until use.

4.2.2.9 Detection of PCR products from the controls by Southern blotting

DNA from the agarose gels was transferred to a nylon membrane (Hybond^{TM} -N^+, Amersham International) using the capillary transfer method as described by Maniatis *et al.* (1982) as follows. After electrophoresis, the gel was soaked in 250 ml

of depurination solution (0.25 M H_2SO_4) for 7 min. After a brief wash in deionised distilled water it was immersed in denaturation solution for 30 min. Then it was briefly rinsed in water and submerged in neutralisation solution for further 30 min. Transfer of DNA to the nylon membrane was achieved by capillary movement of high salt buffer (20x SSC, pH 7.0). A sandwich was assembled as follows: Six pieces of Whatman filter paper were soaked in 20x SSC and placed on top of a plastic support. The gel was placed on top of the wet filter paper and then the transfer nylon membrane (15x7 cm) was placed. A clean pipette was used to roll out any air bubbles present. Six pieces of filter paper of the size of the gel were soaked in 3x SSC and placed on top of the membrane. Then the assembling was finished by placing absorbent paper towels and weight (1kg) on top. The gel was allowed to blot overnight then the membrane was recovered and air dried at 20°C. DNA was fixed to the membrane by placing it under UV light for 5 min (DNA side facing the UV light) and the membrane was ready for probing. Protocols were followed as stated in Boehringer Mannheim's DIG nucleic acid detection kit with slight modifications as explained in section 4.2.2.10. Hybridisation temperatures were raised to 68°C and washing conditions were changed to 0.05x SSC, 0.1% SDS to optimise the results and increase the specificity of the test.

4.2.2.10 Detection of PCR products from tick samples by dot blot hybridisation

Detection, after hybridisation to target nucleic acid, is attained by enzyme-linked immunoassay using an antibody-conjugate (anti-digoxigenin alkaline phosphatase conjugate) as follows. 95-bp PCR amplicons from the 11 different mammal hosts and the samples derived from ticks were denatured in boiling water for 10 min then chilled immediately on ice. 1 µl of each sample was dispensed onto a nylon membrane (10x5 cm) Hybond^{TM} -N+ (Amersham International) positively charged. The membrane was lightly marked with a pencil to identify each sample before dispensing. The DNA was fixed by UV cross-linking for 3 min and the membrane prehybridised at 68°C for 1 h in 20 ml of DNA hybridisation solution containing 5x SSC, 1% blocking reagent, 0.02% SDS and 0.1% N-lauroylsarcosine.

After 1 h the prehybridisation solution was discarded and substituted with 2.5 ml of hybridisation solution containing 1 µl of digoxigenin labelled probe that was also denatured at 99°C for 10 min and chilled immediately on ice before being incorporated to the hybridisation solution. After overnight incubation at 68°C, the membrane was washed twice at low stringency with 2x SSC, 0.1% SDS at 20°C for 10 min in total. It was followed by two higher stringency washes with 0.05x SSC, 0.1% SDS at 68°C for 30 min in total. The membrane was washed for 5 min in buffer 1 containing 0.3% (v/v) Tween 20 then incubated in blocking solution for 30 min (100 ml buffer 2). After another brief wash in buffer 1, the membrane was incubated for 30 min in anti-digoxigenin-AP conjugate diluted 1/5000 in blocking solution. After two further washes in buffer 1 (2x 15 min), the membrane was allowed to equilibrate in detection buffer (20 ml of buffer 3) for 2 min. Then colour substrate solution (10 ml buffer 3, 200µl of vial 4 containing NBT/BCIP stock solution in 67% DMSO) was poured over the membrane and incubated for at least 1 h in the dark. The enzyme catalysed colour reaction was stopped when the desired level of a insoluble blue precipitate to visualise the hybrid molecules was formed by washing for 5 min in sterile distilled water. The membrane was kept wet in sterile distilled water to allow further reprobing or dried and photographed. Buffers composition is summarised in Appendix D.

4.2.3 Results

4.2.3.1 PCR results with degenerate primers to amplify 638-bp fragment of the cytochrome b from sheep, roe deer, bank vole and wood mice

A 638-bp PCR amplicon was obtained from the four vertebrate hosts. Attempts to amplify a fragment of the same length from ticks that were positive for the 95-bp nested PCR were unsuccessful. Kirstein and Gray (1996) determined that big fragments could be amplified only up to day 10 after the blood meal and ticks used in this study were probably moulting, diapausing and questing for up to one year.

4.2.3.2 Nested PCR results using mammal and tick DNA with degenerate primers for the host cytochrome b (95-bp) and specific primers for *E. phagocytophila* (*groE* operon)

A 95-bp fragment was amplified after nested PCR from all vertebrate hosts (Fig. 4.11). 192 field collected ticks were tested individually by nested PCR with specific primers for the host cytochrome b DNA. On average, only 2/5 tested ticks were positive after nested PCR (Fig. 4.12). Interestingly, it was more difficult to amplify host DNA from ticks that were collected from coniferous wood (20/84 positive ticks compared to 20/54 from deciduous and pasture sites) suggesting that ticks on that site where either questing for longer periods or fed on different hosts such as birds whose DNA can not be amplified using these primers (Kirstein and Gray, 1996). Finally, host DNA was obtained from 20 field nymphs from each site.

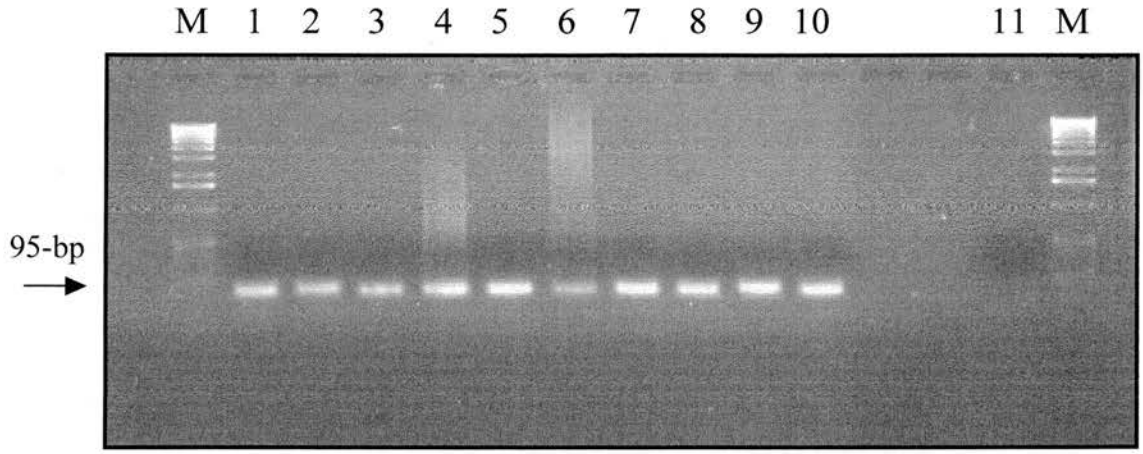


Fig 4.11 Cytochrome b nested PCR to amplify 95-bp products from several vertebrate hosts. The 1.5% agarose gel was stained with ethidium bromide. Arrow indicates position of the 95-bp PCR fragment. M, molecular weight marker (1Kb); Lane 1, rat, Lane 2, rabbit, Lane 3, mice; Lane 4, wood mice; Lane 5, bank vole; Lane 6, sheep; Lane 7, goat; Lane 8, cattle; Lane 9, roe deer; Lane 10, fallow deer; Lane 11, sterile distilled water

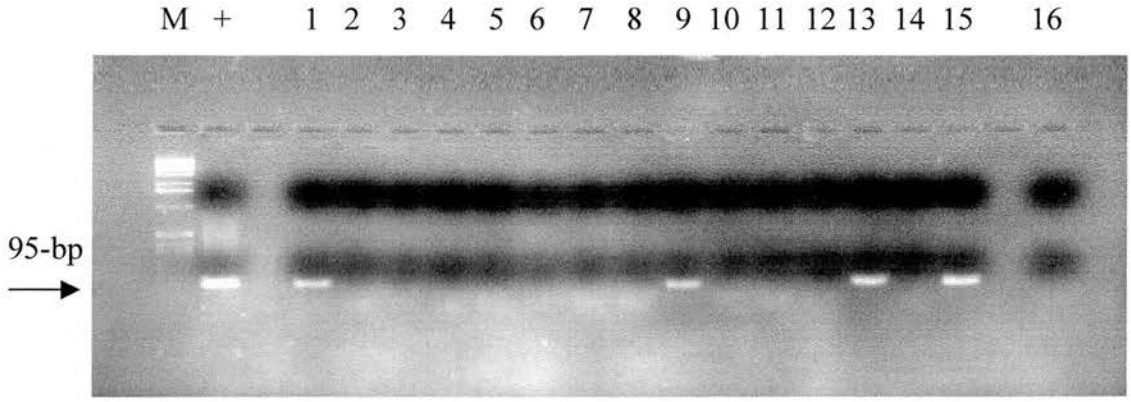


Fig 4.12 Cytochrome b nested PCR on DNA extracted from questing *I. ricinus* nymphs to determine the host on which they fed as larvae. Arrow indicates position of the 95-bp PCR products. M, molecular weight marker (1Kb); Lane +, roe deer spleen sample; Lanes 1-5, tick samples from pasture; Lanes 6-10, tick samples from coniferous wood; Lanes 11-15, tick samples from deciduous wood; Lane 16, sterile distilled water. Lanes +, 1, 9, 13, and 15 are positive showing the expected DNA fragment size

4.2.3.3 Enzymatic digestion of 638 and 95-bp PCR products (RFLP) from sheep, roe deer, bank vole and wood mouse.

After enzymatic digestion, the 638 bp fragment could be used to differentiate rodents from sheep and roe deer, but it did not discriminate between sheep and roe deer samples (Table 4.7). In addition a fragment of this length can not be amplified from the ticks used in this study.

The restriction digestion of the 95-bp PCR product was not able to differentiate between the species of interest, the only host that appeared to differ was wood mouse. It would be necessary to use several different enzymes to distinguish between species making the whole process too slow and complicated.

Table 4.7 Summary of results after restriction digestion of cytochrome b PCR products with several restriction enzymes

	Fragment size (base pairs)			
	638-bp		95-bp	
Enzyme	HaeIII	DdeI	HaeIII	MboII
Sheep	638	521, 90	95	95
Roe deer	638	521, 90	95	95
Bank vole	638	239,170, 133	95	95
Wood mouse	388, 238	398, 140	52, 43	95

4.2.3.4 Cloning, sequencing and design of an internal probe for roe deer based on the 638-bp DNA fragment of the cytochrome b amplified after PCR

A 638-bp fragment from roe deer cytochrome b DNA was successfully amplified, cloned and sequenced. The alignment with previously known sequences indicated a close similarity between the species of interest as shown in Table 4.8. The location of the internal probe is also shown in Table 4.8. The whole 638-bp fragment from roe deer cytochrome b obtained in this study is shown in Appendix 4.3.

Table 4.8 Sequence alignment of the 95-bp DNA fragment from the cytochrome b of roe deer obtained in this study with sheep, cattle, black tailed deer, fallow deer, and mouse known sequences (Irwin *et al.*, 1991). The location of the internal probes is highlighted

		bp
Roe deer	ACCCTGACCCGATTTTTCGCTTTCCACTTTATTCTCCCATTTATCATTGC	50
Fallow deer	---T-A--T-----C--------------------A----------	50
Black-tailed deer	-----A--T-----C----------------C----------C--	50
cattle	-----T--------C-----------T-----C--T----------C--	50
sheep	-----C--------------C--T---------T-------C-----C--	50
mouse	---T----------C--------------C--CT-A----------CAT	50

		bp
Roe deer	AGCACTTGCTATAGTCCATTTACTTTTCCTCCACGAAACAGGATC	95
Fallow deer	---------------A--------A----------G--------	95
Black-tailed deer	----------------------C--------------------	95
cattle	----A----C---------CC----A----------------C--	95
sheep	---C--C--C-----T--CC----C--------------------	95
mouse	G--C--A--A--C--T--CC-C--C--------------------	95

- Indicates same nucleotide as roe deer

4.2.3.5 Southern blot analysis using oligonucleotide probes based on roe deer and mice cytochrome b DNA sequences

Roe deer digoxigenin labelled oligonucleotide probe reacted to rabbit, bank vole, and shrew DNA in addition to deer DNA when using 45°C as the hybridisation temperature. The conditions were changed to 50°C and higher stringency washes (0.05x SSC) aiming to improve the specificity of the probe. However it still reacted to the mentioned hosts. It was decided to use 68°C for prehybridisation and hybridisation and low stringency washes (0.1x SSC) as for the mouse probe. The results were still the same although the probe clearly reacted more strongly to deer DNA. When higher stringency washes were tried, the probe still reacted to non-specific hosts.

After some trials using the mouse oligonucleotide probe (Kirstein and Gray, 1996) digoxigenin labelled it appeared that the probe was not working (as opposed to the roe deer probe of the same length and synthesised by the same manufacturers). Since roe deer probe was not specific enough it was decided to label a 95-bp PCR fragment of Balb/C mice DNA using Boehringer Mannheim DIG DNA labelling and detection kit following manufacturers' instructions. After Southern blotting the results indicated that the mouse probe only recognised mouse DNA, none of the remaining rodents' DNA was hybridised. The membrane was reprobed with labelled roe deer oligonucleotide. The results were as before, the probe recognised roe and fallow deer but also rabbit, shrew and wood mice DNA (Fig. 4.13).

It was decided to use sheep 95-bp labelled PCR product as a probe (68°C hybridisation temperature, low stringency washes). The results indicated that sheep probe reacted to sheep, bank vole and cattle DNA. When using bank vole labelled DNA as a probe it also reacted to sheep and bank vole but not cattle (Fig. 4.14).

When wood mice was used as a probe it strongly reacted to wood mice DNA and to most of the control hosts but not to rat and cattle DNA (Table 4.9).

Table 4.9 Summary of southern blot results using four different mammalian probes and control DNA from 12 different vertebrate species

	Probe				
Control DNA	Balb/c mice	Roe deer	Sheep	Bank vole	Wood mice
Fallow deer		√			x
Roe deer		√			x
Cattle			x		
Goat					x
Sheep			√	x	x
Shrew		x			x
Bank vole			x	√	x
Wood mice		x			√
Balb/c mice	√				x
Rabbit		x			x
Rat					

√	positive reaction
x	non-specific reaction
Blank	no reaction

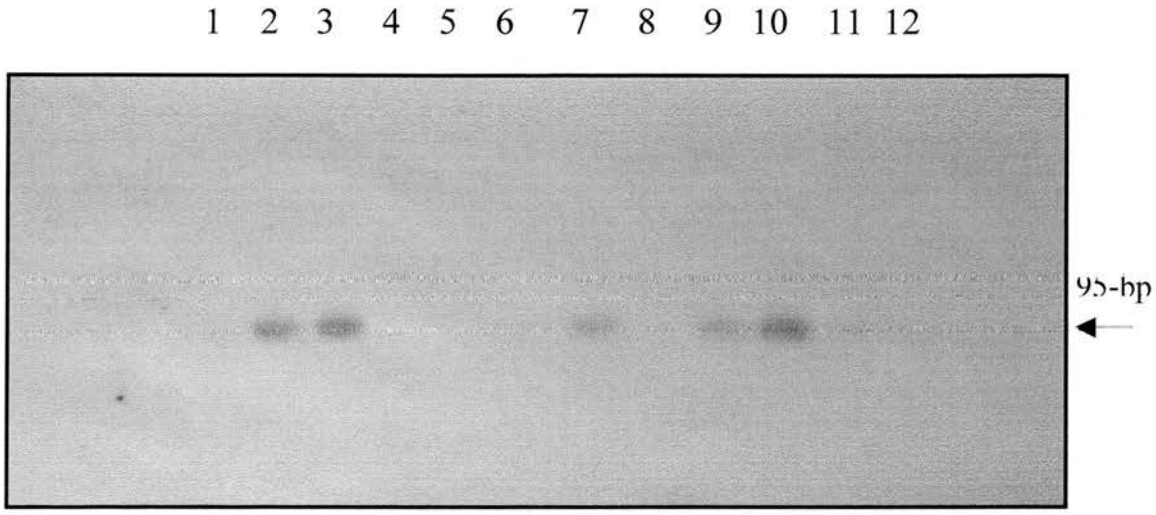


Fig 4.13 Southern blot with control DNA (95-bp PCR fragments from the cytochrome b, (arrow) from several mammals. First the membrane was probed with a digoxigenin labelled 95-bp amplicon derived from mice after PCR, then the membrane was reprobed with a digoxigenin labelled oligonucleotide probe based on roe deer cytochrome b DNA sequence. Lanes 1, sterile distilled water; 2, fallow deer; 3, roe deer; 4, cattle; 5, goat; 6, sheep; 7, shrew; 8, bank vole; 9, wood mice; 10, Balb/c mice; 11, rabbit; 12, rat.

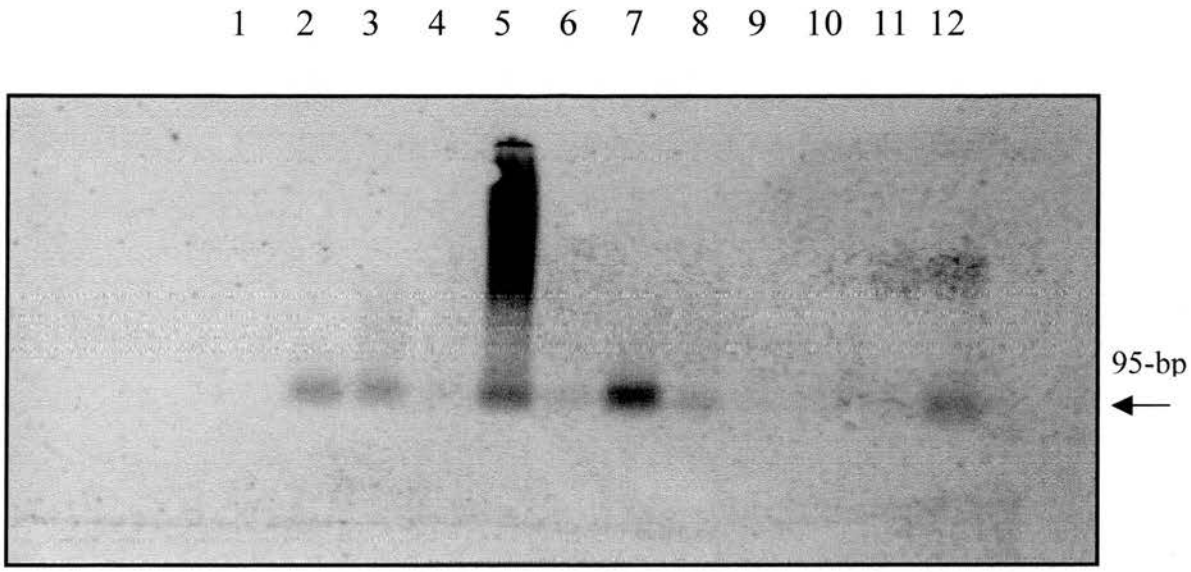


Fig 4.14 Southern blot with control DNA (95-bp PCR fragments from the cytochrome b, arrow) from several mammals. First the membrane was probed with a digoxigenin labelled 95-bp amplicon derived from sheep after PCR, then the membrane was reprobed with a digoxigenin labelled 95-bp amplicon derived from bank vole. Lanes 1, sterile distilled water; 2, fallow deer; 3, roe deer; 4, goat; 5, sheep; 6, shrew; 7, bank vole; 8, wood mice; 9, Balb/c mice; 10, rabbit; 11, rat; 12, cattle

4.2.3.6 Dot-blot analysis of field collected ticks using digoxigenin labelled 95-bp PCR products probes derived from roe deer, bank vole, wood mice, sheep and cattle cytochrome b DNA sequences

Five different probes derived from 95-bp PCR products from bank vole, wood mice, roe deer, sheep and cattle were used in dot blot studies. For each probe a different membrane was used and fresh PCR products from the controls and ticks. Previous results showed that wood mice reacted to most of the hosts but not cattle, bank vole reacted only to bank vole and sheep and roe deer reacted to roe deer, fallow deer, wood mice, rabbit and shrew DNA.

Twenty pasture ticks were tested using five different probes and the 11 controls derived from rat, rabbit, Balb/C mice, bank vole, wood mice, shrew, sheep, goat, cattle, roe deer and fallow deer. Wood mice probe reacted to most controls' DNA. Bank vole probe reacted to bank vole and sheep controls as expected but to none of the ticks suggesting that pasture ticks were not feeding on those hosts. The routine use of acaricides could explain the lack of ticks feeding on sheep. Cattle probe reacted to at least half of the ticks. Although it also reacted to sheep control DNA, this potential host for ticks was discarded because of the previous results using bank vole probe. Because wood mice probe did not react to cattle it suggests that the cattle reacting ticks were truly feeding on those hosts. Roe deer probe reacted to most of the remaining pasture ticks. In summary, pasture ticks were mainly feeding on cattle, then to a lesser extent on rodents and roe deer (Fig. 4.15 and 4.16). Some of the cattle reacting ticks also reacted to roe deer probe suggesting that ticks are feeding on an alternative ruminant host, perhaps red deer.

Three new membranes were used with 20 ticks from a deciduous site and the 11 controls. Three different probes, derived from bank vole, wood mice and roe deer, were used. With bank vole as a probe the results suggested that the ticks were feeding neither on sheep nor on bank vole since the probe did not react to any of the ticks but it reacted to the control sheep and bank vole DNA. Wood mouse probe reacted to most of the controls as before but not to rat and cattle DNA. Most of the ticks also appeared positive suggesting that they were feeding either on wood mice or roe deer.

Although the probe also reacted to rabbit control, this host was discarded because they are not present in the site. The use of roe deer probe confirmed that ticks in a deciduous site were mainly feeding on roe deer and wood mice (Fig. 4.17).

Three more membranes were used for ticks collected from a coniferous site and all the controls with three probes, bank vole, wood mice and roe deer. The results indicated that most of the ticks (17/20) were feeding on roe deer (Table 4.10; Fig. 4.18).

Table 4.10 Summary of dot-blot results using five different mammalian probes and ticks collected from three widespread sites.

		Tick number																			
Site	Probe	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
1	A											√		√			x				
	B																				
	C	√	√			√				x	x	x		x	x		x			x	
	D				√		√	√	√			x			√	√	√	√	√	√	√
	E																				
2	A				√			√					√	√	√	√	√	√	√	√	√
	B																				
	C			√	x	√	√	x	√		√			x	x	x			x	x	x
3	A				√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√
	B																				
	C				x	x	x	x	x	x	x	x	x		x	x	x	x	x		x

1	Pasture	A	Roe deer	D	Cattle	√	Positive reaction
2	Deciduous	B	Bank vole	E	Sheep	x	Non-specific reaction
3	Coniferous	C	Wood mice			Blank	No reaction

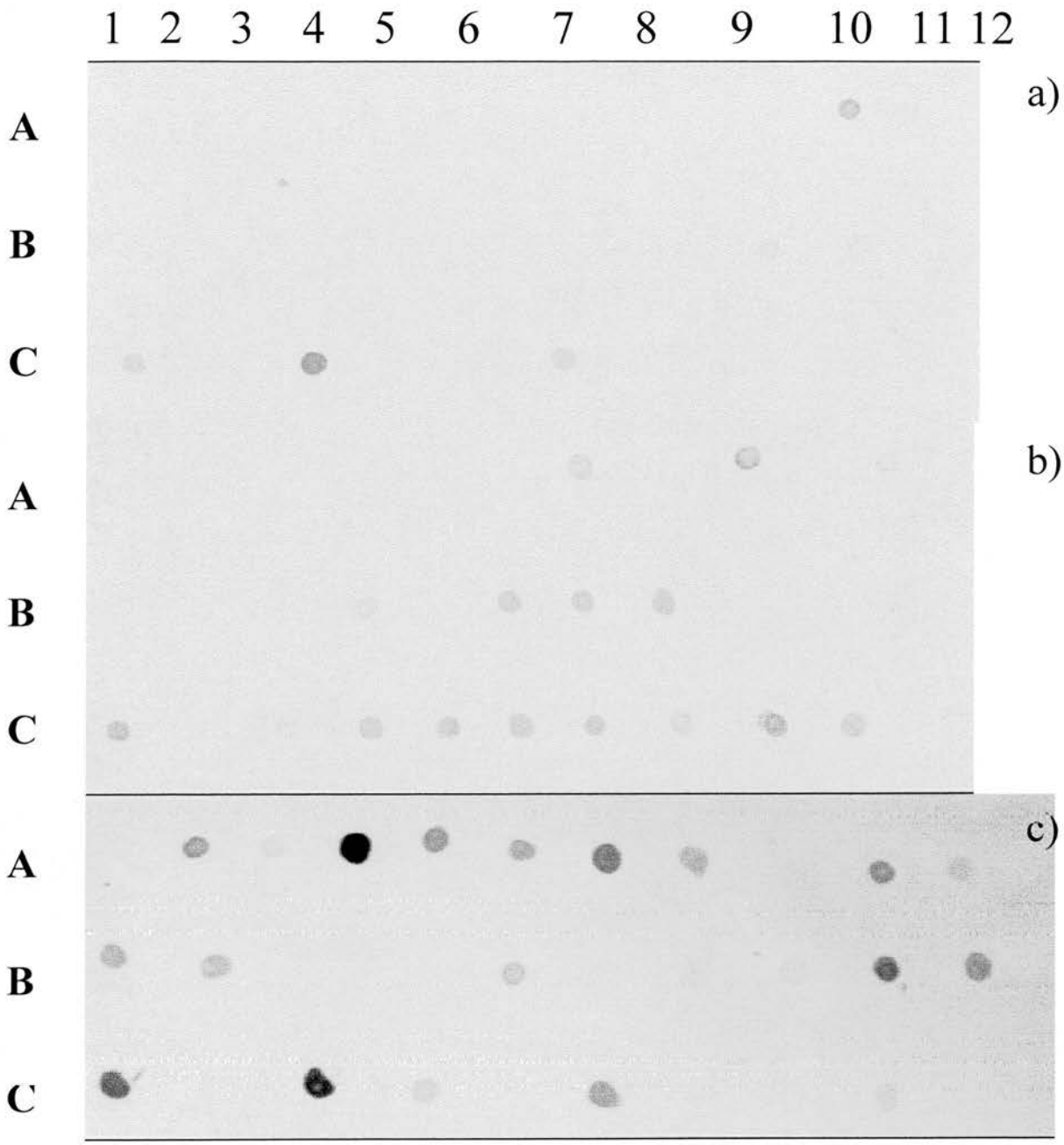


Fig 4.15 Dot-blot analysis of 20 pasture ticks positive after nested PCR for the cytochrome b using three different probes: a) roe deer; b) cattle; c) wood mice. Row A, controls, lane 1, rat; lane 2, rabbit; lane 3, Balb/C mice; lane 4, wood mice; lane 5, bank vole; lane 6, shrew; lane 7, sheep; lane 8, goat; lane 9, cattle; lane 10, roe deer; lane 11, fallow deer; lane 12, distilled water. Rows B and C contain 10 tick derived samples each

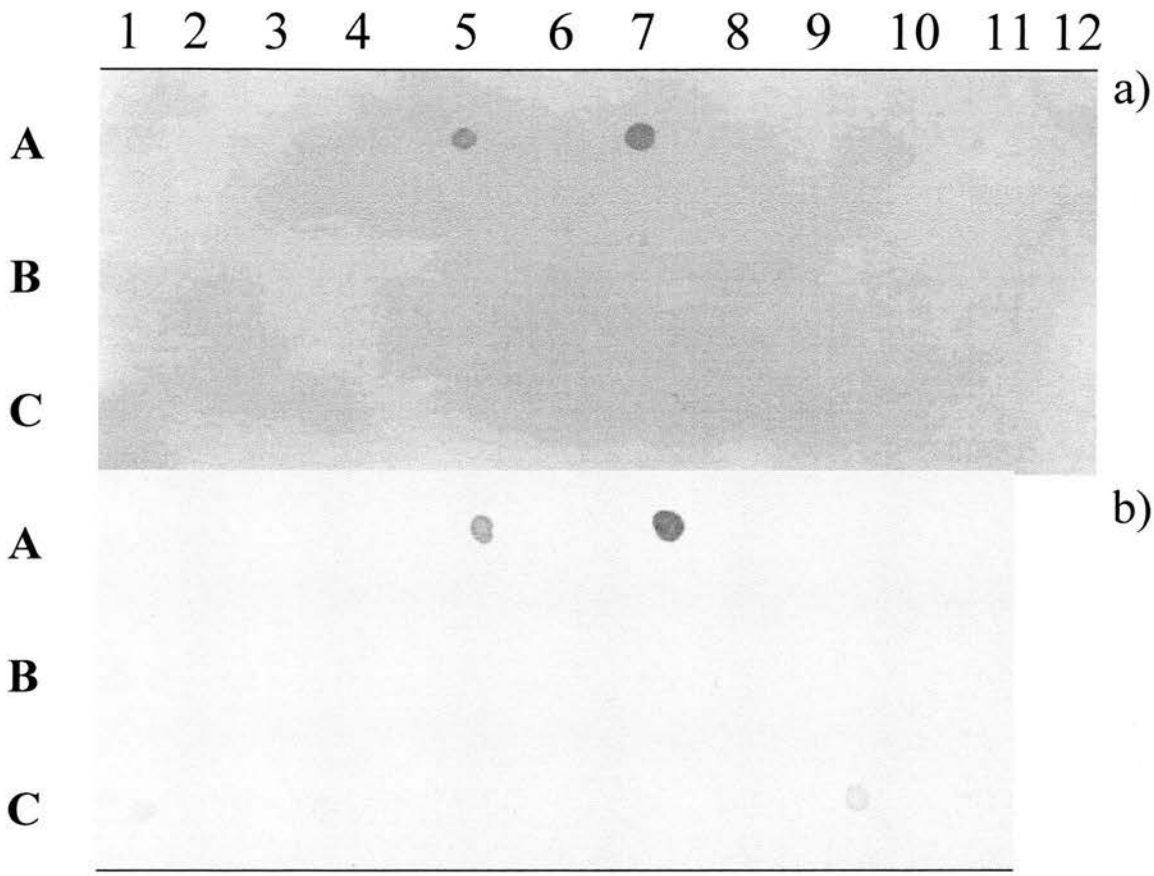


Fig 4.16 Dot-blot analysis of 20 pasture ticks positive after nested PCR for the cytochrome b using two different probes: a) bank vole; b) sheep. Row A, controls, lane 1, rat; lane 2, rabbit; lane 3, Balb/C mice; lane 4, wood mice; lane 5, bank vole; lane 6, shrew; lane 7, sheep; lane 8, goat; lane 9, cattle; lane 10, roe deer; lane 11, fallow deer; lane 12, distilled water. Rows B and C contain 10 tick derived samples each.

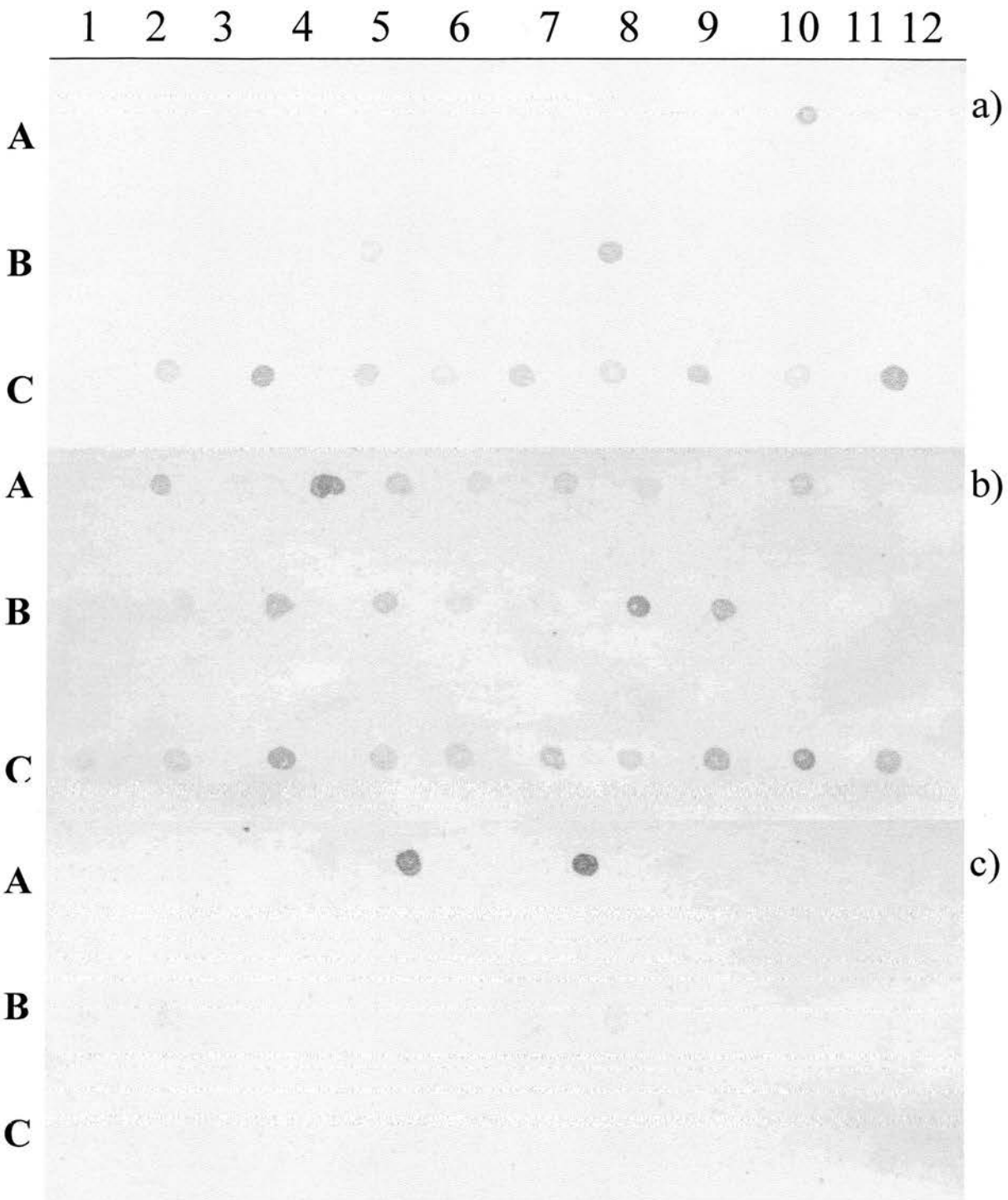


Fig 4.17 Dot-blot analysis of 20 deciduous ticks positive after nested PCR for the cytochrome b using three different probes: a) roe deer; b) wood mice; c) bank vole. Row A, controls, lane 1, rat; lane 2, rabbit; lane 3, Balb/C mice; lane 4, wood mice; lane 5, bank vole; lane 6, shrew; lane 7, sheep; lane 8, goat; lane 9, cattle; lane 10, roe deer; lane 11, fallow deer; lane 12, distilled water. Rows B and C contain 10 tick derived samples each.

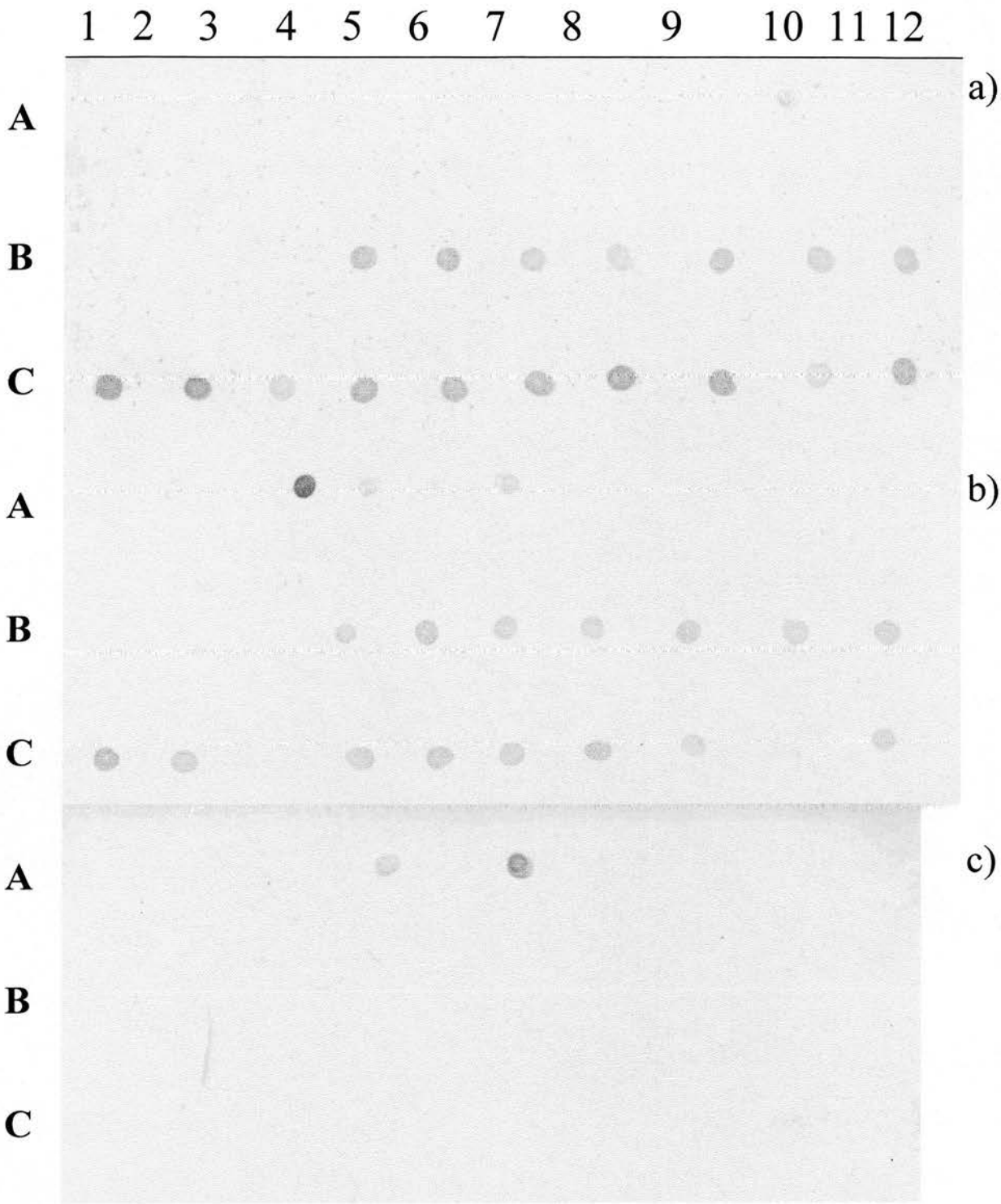


Fig 4.18 Dot-blot analysis of 20 coniferous ticks positive after nested PCR for the cytochrome b using three different probes: a) roe deer; b) wood mice; c) bank vole. Row A, controls, lane 1, rat; lane 2, rabbit; lane 3, Balb/C mice; lane 4, wood mice; lane 5, bank vole; lane 6, shrew; lane 7, sheep; lane 8, goat; lane 9, cattle; lane 10, roe deer; lane 11, fallow deer; lane 12, distilled water. Rows B and C contain 10 tick derived samples each.

4.2.4 Discussion

It is usually believed that sheep are responsible for the maintenance of *E. phagocytophila* infections in both sheep and tick populations, since TBF, caused by *E. phagocytophila,* was first recognised as a disease of sheep (Gordon *et al.*, 1932). Although cattle can also be affected the clinical signs are usually milder and their immunity appears to be sterile (Hudson, 1950). Little is known about the role played as reservoirs by other ruminants, specially wild ruminants, in close contact with sheep flocks. Since transovarial transmission does not occur, ticks can only be infective for the mammal host in the nymph and adult stages. It is of great relevance to investigate the hosts on which tick feed as larvae and nymphs to determine which is the most likely reservoir for *E. phagocytophila* infection.

In this study, *I. ricinus* nymphs from pasture, deciduous and coniferous sites were collected and a method was developed to determine the host in which they fed as larvae. Cytochrome b has been widely used for phylogenetic analysis and it is believed to show enough variation to discriminate between closely related species (Irwin *et al.*, 1991). This study was based on previously described degenerate primers (Kirstein and Gray, 1996) that were able to amplify DNA from most mammalian hosts. However, only small fragments of the cytochrome b (95-bp) were amplifiable from ticks because of the rapid haemolysis of the blood meal in the tick midgut. The study was thus constricted to a small fragment of the gene, which did not appear to be heterologous enough between the species of interest: sheep, cattle, roe deer and rodents. Several probes had to be used in order to obtain any conclusion.

Not all individual ticks whose DNA was extracted gave a positive signal after nested PCR. It has been suggested that avian DNA was not amplifiable with the primers (Kirstein and Gray, 1996). It is also possible the presence of PCR inhibitors in the tick sample, thus impairing amplification, or too much degradation of the blood meal and consequently of the DNA encoding the cytochrome b. The fragment used in this study was only amplifiable up to day 200 after tick engorgement (Kirstein and Gray, 1996) and the ticks in this study may have been questing for longer periods.

Surprisingly, none of the ticks collected from pasture appeared to have fed on sheep. Their blood meal analysis revealed that they were feeding mainly on cattle and, to a lesser extent, on deer and rodents. This result can be explained because of the routine use of acaricides in sheep from that site. Cattle herds, however, are not regularly dipped. Interestingly, humans that seroconverted to the HGE agent in rural areas of England appeared to be associated with cattle rather than sheep exposure (Thomas *et al.*, 1998).

Ticks from coniferous and deciduous sites were found to be feeding mainly on roe deer and rodents, as expected, because neither sheep nor cattle have access to those forests.

The use of restriction enzymes to differentiate between vertebrate hosts does not appear to be promising, at least with the use of this small DNA fragment. It would be necessary to use too many enzymes making the process too complicated. Dot-blot analysis has advantages over restriction digestion. It is quicker once it has been optimised and many samples can be tested at the same time using the same membrane. In this study, only a probe derived from Balb/C mice (95-bp PCR product) appeared to be specific enough, recognising only DNA from Balb/C mice. The rest of the probes cross-reacted with DNA from other species even when the conditions were changed to increase the specificity of the test. It is likely that the identification of cytochrome b regions with higher variability would allow the design of specific probes for each of the species of interest with advantages of quickness over restriction digestion and sequence analysis methods.

The results of the study suggest that larvae can feed and acquire infection from alternative mammal hosts such as cattle, rodents and deer indicating that the maintenance of TBF does not depend on sheep. *Clethrionomys* rodents are known to become resistant to tick feeding soon after the first infestation, however, this does not appear to be the case for *Apodemus* species (Dizij and Kurtenbach, 1995). In our study, none of the ticks seemed to have fed on bank vole suggesting that it is not a preferential host for ticks probably because of their acquired immune responses

against tick feeding. However, the presence of bank voles in the site was not confirmed. Interestingly, sheep also become resistant to tick feeding (Abdul-Amir and Gray, 1987) and they also appeared not to be a preferential host for larvae feeding. This results suggest that hosts that are particularly susceptible to tick-borne diseases may develop an immune response against tick feeding that impairs successful attachment and engorgement and therefore transmission of any pathogen. Thus, exposure to non-infected tick bites induces acquired immunity to tick feeding which indirectly stimulates protection against future pathogen transmission from the same tick species. Bank voles are known to show little immunity to *Borrelia burgdorferi* spirochetes and develop high levels of infection whereas wood mice can control spirochete infection and mount specific immune responses but does not acquire resistance to tick feeding (Kurtenbach *et al.*, 1994). Previous studies also showed that *I. ricinus* ticks, larvae and nymphs, preferred to feed on field mice (*Apodemus*) than on lizards and bank voles (Matuschka *et al.*, 1991) suggesting mice were more effective reservoirs for Lyme spirochetes.

A probe derived from wood mice did not appear to be specific enough, it cross-reacted with most of the controls DNA. Care was taken with PCR amplifications, they were performed several times in order to ensure no DNA contamination between samples occurred. Dot-blot results were consistent each time indicating that the wood mouse probe was too well conserved to be used for blood meal analysis. However, it seemed that *I. ricinus* larvae had a preference to feed on *Apodemus* probably because of the well established tick-host relationship in terms of geological time, which allows the vector to feed repeatedly without undergoing rejection. It appears that despite the wide range of vertebrate hosts available to *I. dammini* they also prefer to feed on *Apodemus* species (Spielman *et al.*, 1985).

PCR amplification with specific primers for the detection of granulocytic *Ehrlichia* in ticks was the next logical step to associate the type of vertebrate host in which ticks fed with the likelihood to be infected. However, since a low prevalence of infection has been found to occur in field collected ticks (see Section 4.1), the study was not pursued.

CHAPTER FIVE, FURTHER DEVELOPMENT OF SEROLOGICAL TESTS FOR GRANULOCYTIC *EHRLICHIA*

5.1 Serological diagnosis of *Ehrlichia (Cytoecetes) ondiri*

5.1.1 Introduction

Bovine petechial fever (BPF) is a sporadic disease of cattle caused by *Ehrlichia (Cytoecetes) ondiri* in the uplands and highlands of Kenya at altitudes between 1500-2800 metres (Haig and Danskin, 1962; Danskin and Burdin, 1963; Walker *et al.,* 1974). It was first described as a haemorrhagic syndrome at Ondiri farm, near Kikuyu, Kenya, in the early 30's. The aetiology was first established by Haig and Danskin in 1962, then Krauss *et al.* (1972) suggested the classification of the bacteria within the genus *Cytoecetes* (*Ehrlichia*) together with *Ehrlichia* (*Cytoecetes*) *phagocytophila*, *Cytoecetes microti* and *E. equi*. *Ehrlichia ondiri* is a small pleomorphic rickettsia-like organism observed within membrane lined vacuoles mainly in neutrophils (Krauss *et al.,* 1972) but also eosinophils, monocytes and endothelial cells thus damaging the capillaries and allowing erythrocytes to escape to form petechiae. The case fatality is high, approaching 50%, and pregnant animals usually abort (Haig and Danskin, 1962; Danskin and Burdin, 1963).

Non-indigenous breeds of cattle (*Bos taurus*) and their crosses with indigenous stock are highly susceptible. The disease also occurs in Sahiwal cattle (*Bos indicus*) which are naturally resistant to heartwater, caused by the closely related rickettsial pathogen *Cowdria ruminantium*. The incubation period ranges from 7 to 21 days (Haig and Danskin, 1962) and the illness is characterised by sudden high fever, agalactia and petechiation of the mucous membranes. Transplacental infection has been found to occur (Danskin and Burdin, 1963). Sheep and goats are susceptible after experimental inoculation, they show fever and parasitaemia but not the haemorrhages characteristic of the disease in cattle (Danskin and Burdin, 1963). Impala (*Aepyceros melampus*), Thomson's gazelle (*Gazella thomsoni*), and wildebeest (*Connochaetes taurinus*) also appear susceptible to experimental infection (Snodgrass *et al.,* 1975). Strain differences have been recorded between isolates of *E. ondiri* that vary in their virulence and antigenicity for cattle and sheep (Snodgrass, 1975). Immunity in cattle to homologous and heterologous challenge appears solid

for at least two years (Danskin and Burdin, 1963). Outbreaks have been associated with grazing paddocks that are only used at the end of dry season when food is limited. Although it has been only described in the highlands of Kenya many other ecologically suitable locations might be endemic in surrounding countries (Walker *et al.*, 1974). Little seasonal variation has been noted in the incidence of the disease (Walker *et al.*, 1974).

There is no proven vector of the disease. The reservoir host is the bushbuck (*Tragelaphus scriptus*), prevalence of carriers amongst them appears to be very high (Snodgrass *et al.*, 1975). Cattle are poor reservoirs of infection (Snodgrass, 1975). Many other wild ruminants and rodents are suspected as reservoirs and arthropods as vectors (Walker *et al.*, 1974). Horses, pigs and dogs appear refractory to experimental infection (Haig and Danskin, 1962). Early research claimed that *E. equi* resembled *E. ondiri* in its morphology when blood smears where examined (Stannard *et al.*, 1969). *Ehrlichia ondiri*, *E. phagocytophila* and *E. equi* are remarkably similar in their morphology and epidemiology of the diseases induced by them to the point that some authors have speculated they may be caused by different strains of the same micro-organism (Snodgrass, 1975). Fever and marked haematological changes are features of TBF and BPF. Lymphopenia is followed by neutropenia and thrombocytopenia also occurs (Danskin and Burdin, 1963). Table 5.1 summarises some of the known details related to the epidemiology of the disease.

Diagnostic tests for the disease have not been fully developed. There are not serological tests or culture methods available. The objective for this study was to develop a test to detect antibodies to *E. ondiri* using *E. equi* and *E. phagocytophila* as surrogate antigens under the evidence of the close relationship between the three pathogens and the common serologic cross-reactions that appear to occur between *Ehrlichia* species.

Table 5.1 Summary of known details about the epidemiology of *Ehrlichia (Cytoecetes) ondiri*, causative organism of bovine petechial fever (BPF)

Natural infection	Experimental hosts	Reservoirs	Suspected vector	Reference
Cattle	Sheep and goats Dogs		*Haematopota* spp.	Danskin and Burdin, 1963
		11 species of rodents studied, all negative	*Rhipicephalus* *Haemaphysalis* Mites	Walker *et al.*, 1974
Cattle Bushbuck	Sheep and goats Impala Thomsons gazelle Wildebeest Buffalo		*Boophilus*	Davies, 1993

5.1.2 Materials and Methods

5.1.2.1 Field sera from cattle and bushbuck (Kenya)

Samples containing antibodies to *E. ondiri* were collected from cattle and bushbuck in Kenya by Dr. D. R. Snodgrass. Samples from Sardinia (Prof. Pennisi) were derived from animals which were suspected to be infected with *Ehrlichia* spp.

5.1.2.2 IFAT and immunoblot studies

IFA test was performed as previously described in Chapter Three. The starting dilution for bovine samples was 1/40 in PBS for IFAT, pH 8.0, and the anti-bovine IgG FITC conjugate (Sigma) was diluted 1/160. Positive samples were titrated using two-fold dilutions.

5.1.2.2.1 Fractionation of *E. equi* crude antigen by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Stock solutions of acrylamide were mixed to obtain 10 % (separating) and 4% (stacking) polyacrylamide discontinuous gels (Laemmli, 1970) (Appendix B) which were used to separate *E. equi* proteins. Gels were cast with a single tooth preparative comb. 100 µl of crude *E. equi* antigen (see section 5.2.2.9) were diluted in 2x reducing sample buffer (Appendix B) then boiled for 10 min to denature the sample and loaded onto the gel (200 µg of protein per gel). Minigels were cast (Miniprotein ® II Dual Slab Cell apparatus, BioRad) then run at 100 volts for 30-45 min until the dye reached the end of the gel. Prestained molecular weight markers (BioRad) were run alongside the samples in the gels.

5.1.2.2.2 Electrophoretic transfer of *E. equi* antigens onto nitro-cellulose membranes

Following electrophoresis the gels were soaked in transfer buffer (48 mM Tris, 39 mM Glycine, 1.3 mM SDS, 20% methanol, Appendix B) for 15 min. Proteins were transferred onto nitro-cellulose membranes with pore size 0.45µm (Hybond C fortified nitro-cellulose paper, Amersham International, UK) using a Transblot SD® (semi-dry) electrophoretic cell (BioRad) for 30 min at a constant 10

v/0.28 A per minigel. After the transfer, nitro-cellulose membranes were rinsed once in PBS and washed twice (5 min per wash) in PBS containing 0.1% Tween 20 (PBST) on a rocking platform. Non-specific binding was blocked by overnight incubation in blocking buffer (5% commercial skimmed milk in PBST). Blots were then washed three times in PBST, vertically cut into 5mm strips and numbered sequentially before being used for Western blotting.

5.1.2.2.3 Immunoblotting

Strips of nitro-cellulose membranes on which antigens had been electroblotted were washed for 15 min and then 2x5 min in PBST on a rocking platform. Sera were diluted 1/50 in blocking buffer and the strips incubated with 2 ml of the dilution for 1 h. Then the strips were washed three times (once for 15 min, then 2x5 min) in PBST and incubated with a 1:500 dilution of anti-bovine IgG horseradish peroxidase (HRP) conjugate (Sigma) in blocking buffer for 60 min. After three washes in PBST as before, the blots were transferred into a container with the colour developing solution (α chloro-naphthol dissolved in ice cold methanol and diluted in TBS supplemented with H_2O_2) and incubated for 5 to 10 min until suitable colour was developed then the reaction was stopped with distilled water. The molecular mass of the bands was determined by using a standard curve prepared by measuring the migration of pre-stained standards (BioRad).

5.1.2.3 Nested PCR to amplify a fragment from the *groEL* gene of granulocytic *Ehrlichia* from Kenyan and Sardinian samples

DNA from serum samples was extracted as follows. 250 µl of sera were aliquoted and centrifuged at 13000 *g* for 30 min. The supernatant was removed and the pellet resuspended in 200 µl of sterile PBS. The sample was further processed using QIAamp Blood Kit (QIAGEN Ltd) according to manufacturers' instructions as described in previous chapters.

Nested PCR was performed using primers HSP354-HSP2165 for the outer amplification and primers HSP534 5'-TGTACTCAATAAGCTCCGTGGTG-3', HSP1324 5'-CTACTCTGTCTTTGCGTTCCTTCA-3' (Appendix 3.1) for the inner

amplification to produce a final 410-bp amplicon. PCR conditions were the same as described previously in Chapter Three.

5.1.3 Results

5.1.3.1 IFAT and immunoblot studies

Table 5.2 summarises the results after IFAT using *E. phagocytophila* and Western blot using *E. equi* as antigens respectively of *E. ondiri* and several *E. phagocytophila* samples obtained from different vertebrate hosts. Reactions to soluble and insoluble fractions of *E. equi* antigen of experimental sheep containing antibodies to *E. phagocytophila* are shown in Fig 5.1. *Ehrlichia ondiri* samples appeared to contain antibodies that recognised both *E. equi* and *E. phagocytophila* antigens. A different banding pattern was evident when TBF-BPF derived samples were compared but all positive samples recognised a 44-kDa band (Fig 5.2), specific protein for granulocytic *Ehrlichia* (Dumler *et al*; 1995, Wong *et al*; 1997, Ravyn *et al*; 1998).

Table 5.2 Summary and comparison of results after testing *E. ondiri* and several samples containing antibodies to *Ehrlichia* by IFAT using *E. phagocytophila* and Western blot using *E. equi* as antigens respectively. Column headed 'Lane' refers to the position of samples in Fig 5.2

Species	Name	IFAT titre	Western blot	Lane
E. ondiri	Muguga	1/400	+	1
	81 2/2	1/800	+	2
	81 9/1	-	-	3
	6848 5/3	1/40	-	4
	6848 2/4	1/1600	+	5
	6773 7/2	-	-	6
	6773 9/3	1/800	+	7
Sardinian *Ehrlichia*	S46	-	ND	
	S123	-	ND	
E. phagocytophila	936 day 0	-	-	11
	936 3 wpi*	1/3160	+	12
	928 day 0	-	-	13
	928 3 wpi	1/3160	+	14
	939 day 0	-	-	15
	939 3 wpi	1/10000	+	16
Ehrlichia spp.	Caithness horse	1/200	+	9
	Penrith cattle	1/800	-	8
	Southampton mare	1/80	-	10

* weeks post experimental inoculation
ND Not done
\+ reaction to 44-kDa antigen
\- no reaction

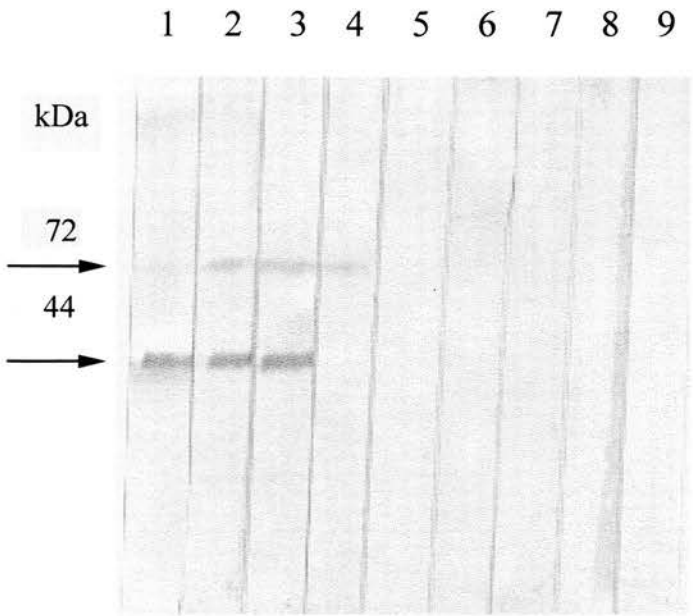


Fig 5.1. Western blot using *E. equi* antigen, soluble and insoluble fractions were tested with experimentally inoculated sheep sera. Lane 1, non-treated antigen; Lanes 2-5, soluble antigen; Lanes 6-9, insoluble antigen. Lanes 1, 2, 3, 6 and 7 were incubated with post-inoculation sera from sheep, 3 weeks after experimental infection; Lanes 4, 5, 8 and 9 were incubated with pre-inoculation sera (Day 0). Arrows indicate the position of proteins of approximate sizes 72 and 44 kDa.

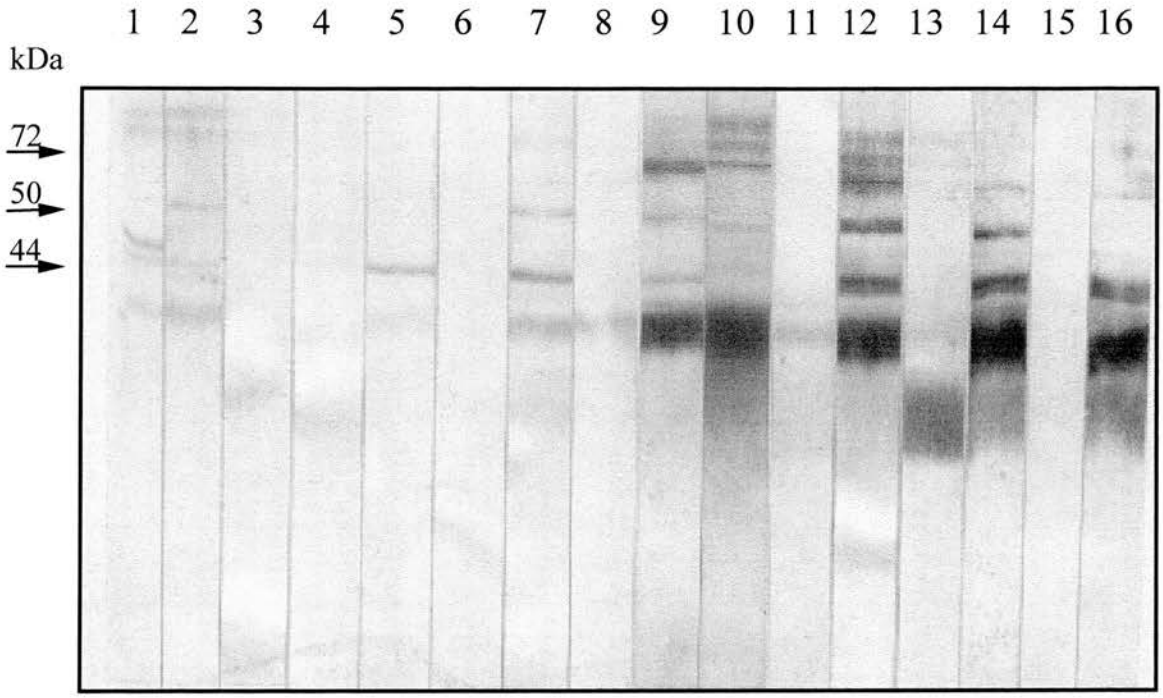


Fig 5.2 Western blot of several sera from domestic ruminants from Kenya which were diagnosed with *E. ondiri* cross-reacting with *E. equi* antigen. Lanes 1-7, *E. ondiri* sera; Lane 8, cattle sample from England; Lane 9, horse from Scotland; Lane 10, Southampton mare; Lanes 11, 13 and 15, pre-inoculation sheep sera; Lanes 12, 14 and 16, 3 weeks post-inoculation sheep sera. Arrows indicate position of bands of approximate sizes 72, 50, and 44 kDa

5.1.3.2 Nested PCR attempts to amplify a fragment from the *groE* operon of Kenyan and Sardinian samples

PCR products were not amplified from either the samples containing antibodies to *E. ondiri* or the Sardinian samples after nested PCR by specific primers to *E. phagocytophila.*

5.1.4 Discussion

Little information is available regarding the epidemiology of BPF. For example, there are not published reports on the presence or absence of humoral antibodies. This lack of interest is in favour of other life-threatening diseases of livestock such as heartwater or East-Coast fever, endemic on the same areas of Africa. However, an understanding of the epidemiology and immmunopathogenesis of *E. ondiri* may contribute to our knowledge of the more severe disease caused by its relative *C. ruminantium.*

It is suspected that *Ehrlichia* species are not as host-specific as it was previously believed. There are emerging zoonotic diseases caused by bacteria closely related if not identical to *E. equi* and *E. phagocytophila* (Chen *et al.,* 1994, Dumler *et al.,* 1995), which are well-known pathogens of horses and ruminants. *Ehrlichia ondiri* could be a strain of *C. ruminantium* or even have derived from *E. canis* to become infective for cattle. There is little evidence of *E. canis* infection in ruminants although serologic relationship is known to exist between *E. canis* and *C. ruminantium* (Kelly *et al.,* 1994). Furthermore, *E. canis* and *C. ruminantium* are genetically 96.8% identical as shown by 16S rDNA sequence analysis (Van Vliet *et al.,* 1992).

Ehrlichia ondiri may be the *Ehrlichia*-like organism, as claimed by recent reports, similar but different to *Cowdria* and contributing to clinical heartwater (Allsopp *et al.,* 1996; Allsopp *et al.,* 1997). In addition, *E. ondiri* also invades endothelial cells and is known to induce lesions in the central nervous system including meningeal congestion and haemorrhages in the dura mater (Danskin and Burdin, 1963). *Cowdria ruminantium* cross-reacts with *E. phagocytophila* (Jongejan

et al., 1989) and antibodies to *E. phagocytophila* can be found in dogs experimentally infected with *E. canis* in the chronic stages of the disease (Waner *et al.*, 1998) suggesting that *Ehrlichia* spp. share common antigenic epitopes that stimulate non-specific antibody responses.

Similarities in the pathology of *E. ondiri*, *E. equi* and *E. phagocytophila* organisms are striking. They all invade cells of the haematopoietic line, mainly neutrophils and eosinophils, and induce marked leucocytic changes due to lymphocytopenia followed by neutropenia and eosinopenia (Krauss *et al.*, 1972). In this study their antigenic relationship is demonstrated for the first time by means of IFAT and confirmed by Western blot.

The evidence suggests that these closely related rickettsial organisms may have evolved from common ancestors. Despite differences in host preference, target cell and vectors, a revision of the classification of the genera within the tribe *Ehrlichieae* appears necessary.

PCR using primers specific for granulocytic *Ehrlichia* did not amplify DNA from *E. ondiri* samples indicating that despite their close antigenic relationship they are genetically more distant. On the other hand, the bacteria may have been absent from blood cells. However the age of the samples has to be also considered. They were stored at -20°C for more than 20 years and they probably contained little intact DNA to amplify. In the case of Sardinian samples, the presence of granulocytic *Ehrlichia* was only suspected by microscopy but not confirmed by means of serology or PCR.

5.2 Development of ELISA for the detection of antibodies to granulocytic Ehrlichia *spp.*

5.2.1 Introduction

Ehrlichia species (Order Rickettsiales) induce serological cross-reactions with closely related bacteria, including *Anaplasma* (Jongejan *et al.*, 1989). *Cowdria ruminantium* and *E. canis* are known to share immunodominant antigens (Kelly *et al.*, 1994). To date there are no highly reliable serological tests for *Ehrlichia phagocytophila* due to the difficulty in cultivating and therefore purifying the organism and because of the variability in antibody production in the different species that are affected by the pathogen. The IFA test (Paxton and Scott, 1989; Hardeng, 1991) is hindered by technical difficulties. It requires skilled personal to perform it and it is also subjective leading to a high probability of producing false negatives or false positives results.

The aim of this work was to develop an ELISA sensitive and specific enough to detect varying antibody levels in the different vertebrate species that appear to be affected by granulocytic *Ehrlichia* pathogens. Both *Ehrlichia equi* and *E. phagocytophila* crude antigens were used.

5.2.2 Materials and Methods

5.2.2.1 Sera from sheep experimentally inoculated with *E. phagocytophila*

Sera from previous experimental inoculations of sheep with *E. phagocytophila* Feral Goat stabilate were used for a preliminary development of ELISA. Their IFAT titre was previously determined ranging from 1/360 to 1/10000 (Paxton, personal communication, see Appendix 5.1) depending on the time after experimental inoculation.

5.2.2.2 Samples from experimental deer that tested negative to louping-ill virus (Moredun Research Institute)

One hundred and forty five samples from several deer species, mainly roe deer, that tested negative for antibodies to louping-ill virus were kindly provided by Irene Pow (Moredun Research Institute). They were used to check the reliability of the anti-sheep conjugate for deer samples and to help determine cut-off points for deer species. There was no information available regarding the history of the animals or exposure to ticks. The prevalence of antibodies to *E. phagocytophila* was expected to be low because of the lack of exposure to louping-ill virus which is also transmitted by *Ixodes ricinus* ticks (MacLeod and Gordon, 1932).

5.2.2.3 Feline samples

Fifty one domestic cat samples obtained from the Department of Veterinary Pathology, University of Glasgow were tested by cELISA. The samples were used in previous studies to determine the presence of antibodies to the feline inmunodeficiency virus (FIV). No details regarding the history, exposure to ticks or clinical signs were available for the samples.

5.2.2.4 Canine samples

Seventy three dog samples from the area of Edinburgh that were submitted to the Veterinary Clinical Laboratory, Royal Dick School of Veterinary Studies, University of Edinburgh and tested negative by IFAT (Chapter Three) were included to check the reliability of cELISA and determine cut-off points for this species. 17 canine samples from Edinburgh and Caithness areas that tested positive by IFAT (Chapter Three) were also studied.

5.2.2.5 Field collected roe deer plasma and sera

One hundred and two plasma and serum samples collected from roe deer and examined by IFAT in a previous chapter (Chapter Three) were used to confirm their results by ELISA.

5.2.2.6 Peptide ELISA

Twelve peptides representing the homologues of *Cowdria ruminantium*, *E. chaffeensis*, HGE and *E. phagocytophila* (Table 5.3), were designed according to the known amino acid sequences of *groE* gene major antigenic regions as identified in previous studies (Sumption *et al.*, 1997). Peptides were synthesised by Chiron Mimotopes (Chiron Technologies Pty Ltd, Australia). Sheep sera with antibodies to *E. phagocytophila*, goat sera with antibodies to *C. ruminantium* and dog samples with antibodies to *E. phagocytophila*, *E. chaffeensis* and *E. canis* were tested against the peptides.

ELISA using biotinylated peptides was developed as follows. A reaction volume of 100 µl was used in 96 well microplates (Immulon I, Dynatec Microtiter®). Plates were coated overnight with streptavidin solution (1 µg/ml) and allowed to evaporate to dryness at 37°C. Washes (4x) with phosphate-buffered saline (PBS) containing 0.1% Tween 20 (PBST) followed. Plates were blocked with 1% casein in PBS (1g casein/ 100 ml PBS) for 1 h then washed 4x as before. 1/1000 dilutions of 1 µmol peptide solution in PBST/AZ (0.1% Tween 20, 0.1% sodium azide) were added and the plates incubated for an hour. Sera reactivity to streptavidin was absorbed prior to the test by incubating the samples for an hour diluted in PBST/AZ (1/800 dilution for sheep and goats, 1/400 for dog samples) in streptavidin coated wells (that contained double concentration of streptavidin. Blocking plates were washed 4x before the addition of sera). Test plates were washed 4x as before then 100µl of absorbed sera were added and the plates incubated for an hour. After further washes anti-species peroxidase conjugate (Sigma) at a dilution of 1/8000 (1/4000 for anti-dog conjugate) in PBST/casein (0.1% Tween 20, 0.1% casein) was dispensed and the plates incubated for 1h. After final washes colour was allowed to developed with 100 µl/well of 3,3',5,5'-tetramethylbenzidine (TMB, Kirkegaard & Perry). The enzymatic reaction was stopped after 4-10 min of incubation at room temperature by adding 100 µl/well of 0.2M H_2SO_4. The optical density (OD) was measured at 450 nm on a Multiskan^{TM} spectrophotometer (Version 2.03, Labsystems, UK).

For each sample the % reaction was measured as follows:

$$\% \text{ reaction} = \frac{\text{mean peptide OD-mean no peptide OD}}{\text{mean biotin control OD}} \times 100$$

Table 5.3 Amino acid sequence of peptides representing the homologues in several *Ehrlichia* species of the major antigenic regions of *Cowdria ruminantium groEL* gene

Peptide	Species	Amino acid sequence of 20-mer peptides	Label
13	*Cowdria*	PEDPLALAIANIIAQSASQC	13Cow
	aoHGE	--E---A---S--T------	13HGE
61	*Cowdria*	CQIRMQIDNSTSDYDKEKLQ	61Cow
	aoHGE	N--KA--E--S--------R	61HGE
	E. chaffeensis	N--K---EA----------R	61cha
	E. canis	N--KV--ES----------R	61canis
82	*Cowdria*	QNDKELIFNVDVTNFANAFT	82Cow
	aoHGE	-------Y---TM-Y-----	82HGE
	E. chaffeensis	-----------TM-------	82cha
89	*Cowdria*	FMTLNAIVVDIPSKDDNSAAGGAGM	89Cow
	aoHGE	------V---V---N-AAG-	89HGE
	E. chaffeensis	-----V-----AN-GA-GMG	89cha
		(overlapping sequence)	

\- indicates same amino acid as *Cowdria ruminantium*

5.2.2.7 Biotin labelling of anti-*E. phagocytophila* IgG polyclonal antibodies

Sera from sheep 935, 3 weeks post-inoculation, was selected for biotin labeling because of its high titre of IgG polyclonal antibodies (>1/10000) after experimental inoculation with *E. phagocytophila* Feral Goat blood stabilate as determined by IFAT. IgG polyclonal antibodies (150000 Daltons) purification followed. 0.54 g of $(NH_4)_2\ SO_4$ (ammonium sulphate) were added slowly to 2 ml of serum stirring constantly at room temperature. The mixture was incubated for 1h at 20°C in a universal tube on a roller mixer then centrifuged at 5000 *g* for 10 min at 4°C. The supernatant was discarded and the pellet resuspended in 400 µl of sterile

PBS, pH 7.2, and dialysed overnight against distilled water to remove any remaining $(NH_4)_2$ SO_4. 1/2 inch dialysis membranes (Sigma) were used against 100x volume of dH_2O. Membranes were treated prior to dialysis as follows. They were boiled 2x for 5 min in 150 mM $NaHCO_3$, 5mM EDTA (using fresh buffer each time) with washes in dH2O in between. The IgG solution was recovered from the membrane and the protein concentration determined by the bicinchronic acid method (BCA Protein Assay kit, Pierce, USA) with protocols described by manufacturers. Briefly, protein standards were prepared by diluting the stock bovine serum albumin (BSA) provided with the kit in PBS to obtain a range between 100 and 1200µg/ml. Ten µl of the samples were added to 200 µl of working reagent (1 part of reagent B: 50 parts of reagent A) in a microtitre plate. Incubation followed for 30 min at 37°C. The optical densities (OD) were read at 570 nm using a microtitre plate reader (Multiskan Plus™, Labsystems, UK). Protein concentrations of the Ig were determined after plotting a standard curve of the OD values of BSA standards.

For biotin labelling, purified IgG was diluted in 0.1 M bicarbonate buffer (pH 9) to 2 mg/ml. Ten milligrams of biotinamidocaproate (Sigma) were dissolved in 1 ml of dimethyl sulphoxide (DMSO, Sigma) and 30 µl of biotin solution were added to each ml of IgG. The sample was left mixing at 20°C for 3 h. Then it was dialysed overnight at 4°C against PBS (100x the volume to be dialysed, v/v). Finally 50 µl of 1% sodium azide was added to the Biotinylated Ig (BIg) and the solution was stored at -20°C until use.

5.2.2.8 Monoclonal antibodies (MAb)

Three different monoclonal antibodies (supernatant) raised from mice were obtained from Dr. Dana Ravyn (University of Minnesota): R1B10, R5A9, R5E4. Their titres were determined by IFAT as previously described (Chapter Three) using *E. phagocytophila* infected neutrophils as antigen and the results compared to the IFAT titres obtained using *E. equi* and HGE as antigens. Anti-mouse FITC conjugate (Sigma) was diluted at 1/32 in PBS for IFAT, pH 8.0.

5.2.2.9 Processing of *E. equi* antigen grown in tick-cell culture to use in serological tests

Ehrlichia equi antigen grown in tick-cell culture was kindly provided by Dr. Ulrika Munderloh (University of Minnesota). The lyophilised antigens were reconstituted in 400 µl of PBS containing 0.02% sodium azide then inactivated overnight. 100 µl of infected tick cells were aliquoted and centrifuged at 10000 *g* for 10 min. The supernatant was removed and discarded. To solubilise the antigen 50 µl of lysis buffer (Appendix A) containing 1 µl of protease inhibitor cocktail (cØmplete™, Mini EDTA-free, Boehringer Mannheim) were added and the sample was incubated at 37°C for 30 min. The sample was dispersed by vigorous pipetting then reincubated at 37°C for 15 more min and centrifuged at 16000 *g* for 30 min. Before centrifugation 10 µl were removed and stored at -20°C to use as crude antigen for SDS-PAGE followed by western blot. After centrifugation the supernatant was removed and stored at -20°C until use for ELISA. The pellet was resuspended with 50 µl of lysis buffer and stored also at -20°C.

5.2.2.10 Cytocentrifuge preparation of slides

For the preparation of slides, 50 µl of the suspensions were spun at 150 *g* for 5 min using a cytocentrifuge (Cytospin, Shandon). After centrifugation, the sample spread on the smear was air dried and fixed in methanol for 5 min then stained with Giemsa. The slides were examined by light microscopy under a 100x objective lens.

5.2.2.11 Indirect and competitive ELISA using tick-cell culture *E. equi* as antigen

5.2.2.11.1 Competitive ELISA with a biotinylated IgG (BIg) antibody to *E. phagocytophila*

The competitive ELISA differs from the indirect assay in that the presence of anti-*Ehrlichia* antibodies in the sample blocks the binding of the monoclonal or BIg (biotinylated competitor) antibodies to specific epitopes leading to a proportional reduction in ELISA signal.

BIg was first titrated against *E. equi* antigen (supernatant) diluted 1/2000 in carbonate/bicarbonate buffer (Appendix A). Plates were coated overnight at 4°C with the diluted antigen. The extravidin-peroxidase (Sigma) conjugate dilution was 1/2500 in PBST. Three different sera (Positive samples with high and low titre, and negative sera to *E. phagocytophila*) and PBS were tested at several dilutions (0-20-50%). BIg was diluted 1/20 to 1/1280 in PBS to optimise the amount of BIg to mix with the serum.

5.2.2.11.2 Competitive ELISA using monoclonal antibodies raised against *E. equi*

The MAb with the highest titre (R5A9) was chosen since the other two did not give any or very low reaction by IFAT or ELISA (Table 5.4, Fig 5.3.a). To determine the optimum conjugate and MAb dilutions to use, plates were coated overnight at 4°C with 1/2000 dilutions in carbonate-bicarbonate buffer of *E. equi* antigen. Serial dilutions of MAb in PBST were made from 1/2 to 1/256. The conjugate (Goat anti-mouse IgG horseradish peroxidase, Sigma) dilution ranged from 1/2000 to 1/20000 in PBST. Highest responses were obtained when the MAb dilution ranged from 1/2 to 1/8. Three different sera (High, low titre, and negative sera to *E. phagocytophila*) and PBST as a background control were tested at two different dilutions (20 and 50%) and three different MAb dilutions (1/2, 1/4/, 1/8).

cELISA using samples from the different vertebrate species was optimised as follows. Polystyrene flat bottomed ELISA plates (Immulon-I, Dynatech laboratories, USA) were coated overnight at 4°C with 100 µl per well of *E. equi* antigen (1/2000

to 1/8000 dilutions in carbonate-bicarbonate buffer). The excess of antigen was removed by flicking the plates and hand washed gently 5 times with PBS containing 0.1% Tween 20 (PBST). Then the plates were filled with 50 µl/well of serum diluted in PBST. Each serum sample was tested in duplicate. 50 µl of ½ dilutions of monoclonal antibody R5A9 were added immediately after the serum. Plates were incubated at 37°C for 2 hours on an orbital shaker (Vari-shaker, Dynatech), then washed 4 times in PBST. 100 µl of conjugate diluted in PBST was added to each well and incubated at 37°C for 1h. Further washes (4 x) with PBST followed. Then each well was filled with 100 µl of substrate TMB (Kirkegaard & Perry). Colour was allowed to develop for 5-10 min. Reactions were stopped with 100 µl/well of H_2SO_4 (0.2 M) and the absorbance was immediately read at 450 nm wavelength by an ELISA plate reader (Multiskan Plus™, Labsystems, UK). The readings were converted to percent inhibition as follows.

$$\% \text{ inhibition} = \frac{\text{mean OD PBS- mean OD sample}}{\text{mean OD PBS}} \times 100$$

All samples were scored as positive or negative for granulocytic *Ehrlichia* depending on the % inhibition values.

Table 5.4 IFAT titre of monoclonal antibodies raised to *E. equi* using three different antigens. MAb R5A9 that gave the highest titre to *E. phagocytophila* was subsequently used in ELISA

		Type of antigen; Inverse dilution of titre		
Monoclonal antibody	Isotype	HGE-2*	*E. equi**	*E. phagocytophila*
R5E4	IgG1	256	16	<40
R5A9	IgG2a	128	64	80
R1B10	IgG3	256	16	40

* Results kindly provided by Dr. D. Ravyn

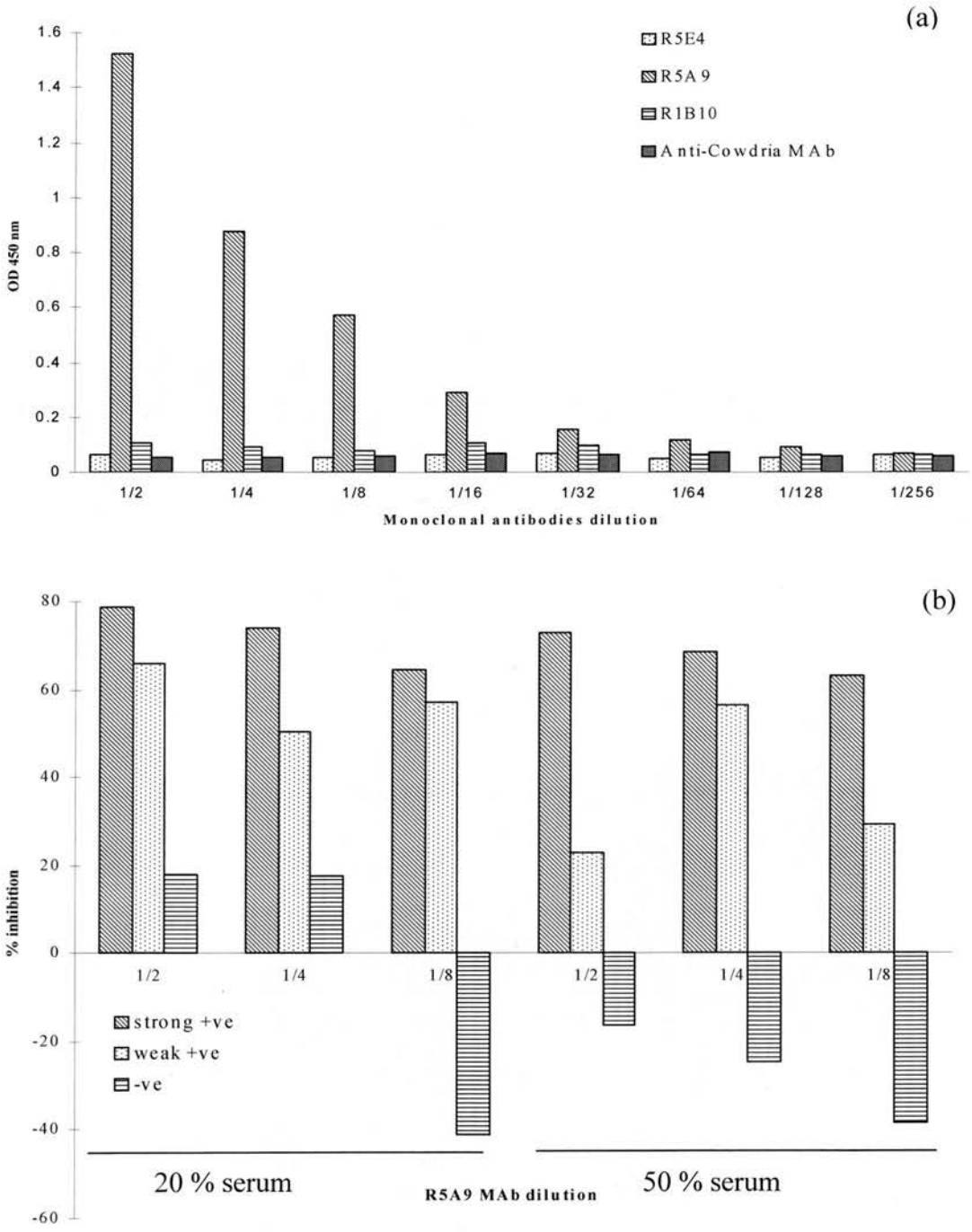


Fig 5.3 (a) Titration of three monoclonal antibodies raised to *E. equi* and a monoclonal antibody raised to *Cowdria ruminantium* against *E. equi* antigen 1/2000 dilution. Only monoclonal antibody R5A9 reacted. (b) Titration of monoclonal antibody R5A9 vs. high, low and negative serum to *E. phagocytophila* at 20 and 50% dilutions using *E. equi* as antigen, 1/2000 dilution

5.2.2.11.3 Indirect ELISA with *E. equi* as antigen

Polystyrene flat bottomed ELISA plates (Immulon-I, Dynatech laboratories, USA) were coated overnight at 4°C (covered with plastic film to prevent evaporation) with 100 µl per well of *E. equi* antigen (supernatant) diluted in carbonate-bicarbonate buffer (1/2000 to 1/8000 dilutions). The excess of antigen was removed by flicking the plates and hand washed gently 5 times with PBST. Then the plates were filled with 100 µl/well of serum diluted in PBST, PBST containing 2% normal rabbit serum or PBST containing 2% commercial skimmed milk. Each serum sample was tested in duplicate. The plates were incubated at 37°C for 2 hours on an orbital shaker (Vari-shaker, Dynatech), then washed 4x in washing buffer (PBST). 100 µl of 1/10000 to 1/20000 dilutions in PBST of donkey anti-sheep IgG HRP conjugate (Sigma) was added to each well and incubated at 37°C for 1h. Further washes (4 x) with PBST followed. Then each well was filled with 100 µl of substrate TMB (Kirkegaard & Perry). Colour was allowed to develop for 5-10 min. Reactions were stopped with 100 µl/well of H_2SO_4 (0.2 M) and the absorbance was immediately read at 450 nm wavelength by an ELISA plate reader (Multiskan Plus^{TM}, Labsystems, UK). iELISA cut-off level was determined with sera from uninfected sheep populations used in previous experiments. The results were corrected by a factor on the reader (mean of the positive control of reference plate/mean of the positive control being read) to ensure that all plates were compared at the same level.

To confirm the observed reactions where specific to *E. equi* and not to tick-cell components, an ELISA plate was incubated overnight at 4°C with 1/1600 dilution of non-infected tick cells (kindly provided by Leslie Bell-Sakyi) in carbonate-bicarbonate buffer that was previously determined to give the highest background reactions in an independent test. iELISA followed as previously described above.

5.2.2.12 ELISA using native *E. phagocytophila* antigen purified on Percoll density gradients

To date there are no long-term cultivation systems for *E. phagocytophila* thus hindering the development of specific serologic tests and the understanding of the molecular biology and antigenic composition of the bacteria. In addition, despite the close relationship and serologic cross-reactions observed between granulocytic *Ehrlichia*, higher antibody levels are expected against specific antigens. The aim of the study was to isolate pure *E. phagocytophila* as elementary bodies, free from sheep blood components to use as antigen for ELISA, based on density gradient centrifugation on Percoll. This is a medium for making density gradients which consists of particles of silica of varying size which are coated with polyvinylpyrolidone and suspended in water. It can be diluted to give gradient steps of predetermined density or formed into continuous gradients at high *g* force in an ultracentrifuge.

5.2.2.12.1 Experimental inoculation of sheep with *E. phagocytophila*

Four female sheep (2 year-old Blackface) were intravenously inoculated with 1 ml of 1/10 dilution in PBS of the *E. phagocytophila* Ehr/8 blood stabilate isolated from Galloway (Scotland) (Chapter Seven). The peak of parasitaemia in experimental sheep was anticipated to occur from day 6 after inoculation (see Results). 20 ml of peripheral blood was collected in heparin tubes from the sheep from day 6 for the isolation of *E. phagocytophila*. Sheep were daily monitored and their clinical signs and rectal temperatures recorded.

5.2.2.12.2 Antigen isolation from whole blood using Percoll density gradients

Ehrlichia phagocytophila are known to have high buoyant densities (Woldehiwet *et al.*, 1991). Several approaches were tried in order to optimise the isolation and purity of the final antigen to use in ELISA. The method was first developed by using a double centrifugation process in Percoll gradients (Pharmacia Biotech). The aim was to isolate the granulocytes from whole blood, after centrifugation on a shallow Percoll gradient, then centrifuged on a second, steeper

gradient of Percoll after cell disruption to isolate pure *Ehrlichia*. Self-forming gradients of Percoll were thus used in order to isolate the pathogen in a band which correlated with density marker beads. A stock isotonic Percoll (SIP) solution was prepared by adding nine parts of Percoll (v/v) to 1 part (v/v) of HBSS (Hanks Balanced Salt Solution). Density marker beads (Pharmacia Biotech) were reconstituted with 1 ml of sterile distilled water and allowed to swell overnight. Their densities ranged from 1.018 to 1.138 g/ml for Percoll in saline solution and they were used as external markers. 70% SIP solution in HBSS self-forming gradients were prepared. Marker beads were added to one of the tubes containing identical gradient material to use as a control tube. Centrifugation followed at 20000 *g* for 20 min at 20°C. Top 5 ml of the gradients was removed and replaced with fresh blood. Centrifugation followed at 1000 *g* for 5 min. The top layer of plasma and thrombocytes was removed and the volumes replaced with 40% SIP then centrifuged at 1000 *g* for 20 min. After centrifugation the gradient appeared too dense at the bottom. The erythrocytes were not at the end of the tube as expected and because there was no clear separation between different kinds of leukocytes the whole layer containing white blood cells was collected and lightly disrupted in a 10 ml tissue grinder. The resultant solution after cell disruption was mixed with 70% SIP and ultracentrifuged at 30000 *g* for 30 min. At the end of the process in the control tube containing the marker beads there was a coagulated band of material (including a mixture of beads of different densities) in the same position where there was a red band of material in the rest of the tubes. There were no beads at the bottom, below the coagulated band (1.074 g/ml). Fig 5.4.a shows the migration of density marker beads in the first (self-forming) and second (after incorporation of the sample) ultracentrifugations. Four fractions (gradients) were collected onto universals: top diffuse white band between blue and red beads (1.051 g/ml), middle dark band just below the blue beads (1/059 g/ml), lower dark band (1.102 g/ml), and bottom clear fraction (with no apparent pellet). Universal tubes containing the four different fractions were filled with sterile PBS and centrifuged at 1500 *g* for 20 min, the supernatant was poured off and the pellet resuspended in 500 µl of HBSS/BSA. Cytocentrifuge slides were made for each sample and stained with Giemsa and

pararosaniline dye. Table 5.5 summarises the appearance of the cytospins after examination by light microscopy.

Table 5.5 Appearance of cytospins after first and second ultracentifugations in gradients of Percoll

Interface from first centrifugation	Mixture of monocytes, lymphocytes and neutrophils
Top diffuse white band	Mainly lymphocytes, some monocytes and neutrophils
Middle dark band	Mainly neutrophils, most of them undisrupted, some free *Ehrlichia*
Lower dark band	Mixture of the three types of WBC
Bottom clear band	Mainly neutrophils, some lymphocytes and monocytes, also erythrocytes and debris

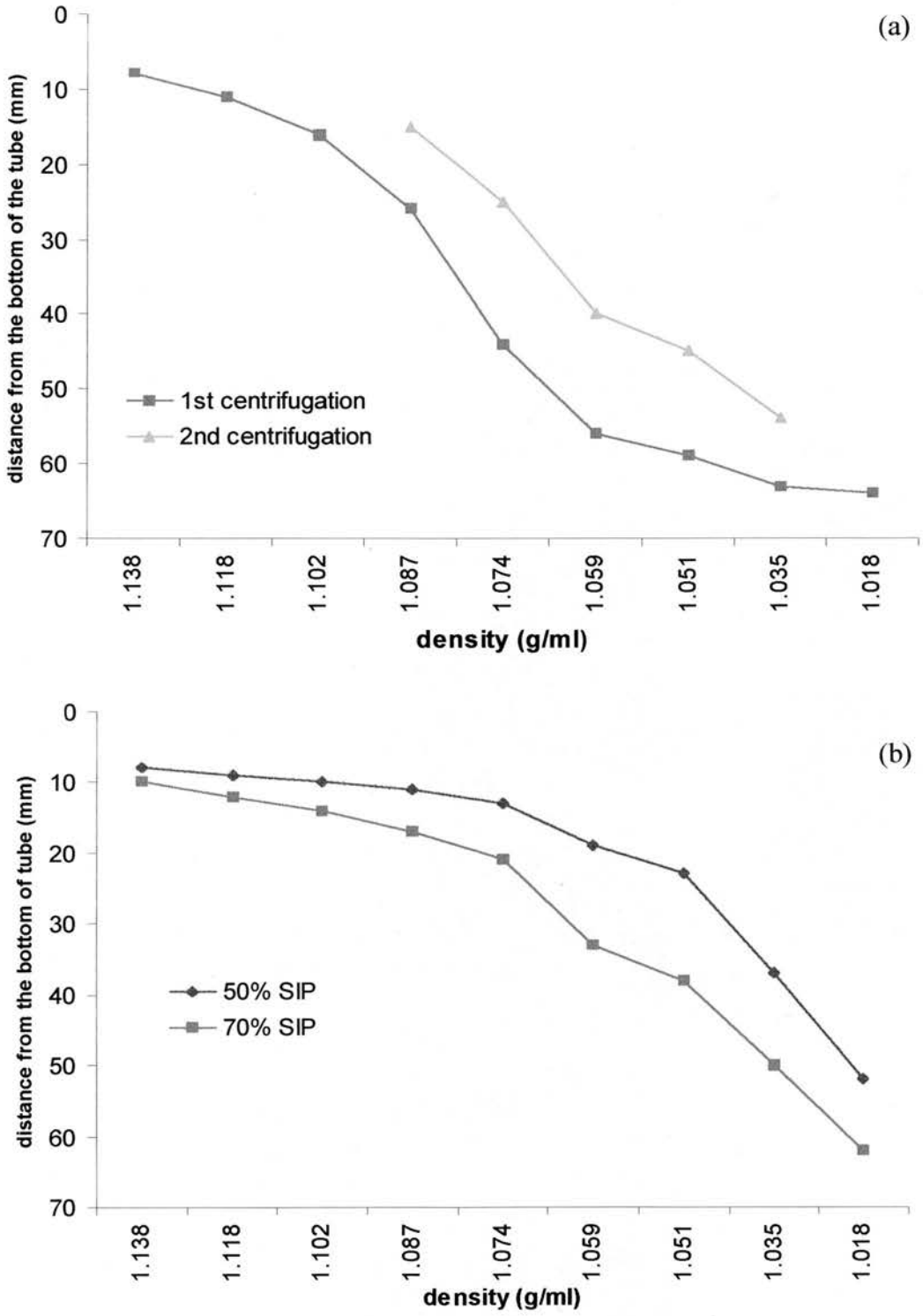


Fig 5.4 (a) Relative migration of density marker beads after centrifugation in self-forming gradients of Percoll at 20000 *g* for 20 min and at 30000 *g* for 30 min after addition of the sample. (b) Relative migration of density marker beads after centrifugation in 50 and 70% SIP step forming gradients of Percoll

5.2.2.12.3 Antigen isolation from infected neutrophils using Percoll density gradients

Further separation of neutrophils from blood components appeared to be necessary in order to purify *E. phagocytophila* as recommended by different studies (Woldehiwet *et al.*, 1991). Ficoll-paque (Pharmacia) was used to isolate granulocytes because the method is designed to separate the lymphocytes from the rest of white-blood cells. Fresh blood collected in heparin was centrifuged at 1000 *g* for 15 min then the buffy coat was removed and resuspended in 3 ml of PBS. The remaining erythrocytes were lysed with the addition of distilled water. The isotonicity was restored with PBS then the sample was centrifuged at 1500 *g* for 5 min to pellet the leukocytes. The supernatant containing lysed erythrocytes was discarded and the pellet resuspended in PBS. The sample containing the leukocytes was poured slowly on top of the Ficoll contained in a universal tube to produce an interface. The universal tube was centrifuged at 2000 *g* for 35 min at 15°C. The pellet, which was expected to contain the granulocytes separated from mononuclear cells, was washed in PBS, then centrifuged at 1500 *g* for 10 min at 4°C. The final pellet was resuspended in 3 ml of PBS. Half of the sample was reconstituted in 20 ml of PBS and nebulised for 3 min, then it was centrifuged at 1500 *g* for 10 min. The supernatant should contain free *Ehrlichia* and the pellet the disrupted granulocytes. The other half of the sample was processed using a tissue grinder as before. Examination of cytospins revealed that neither the nebulising nor the grinding processes disrupted the cells completely. A mixture of granulocytes and lymphocytes was found in Ficoll pellet indicating that the separation of granulocytes from lymphocytes was not as good as expected so the use of Ficoll-paque was discarded.

The method was further developed as follows. Whole blood collected in heparin tubes was processed using the method described by Carlson and Kaneko (1973) with some modifications. Briefly, samples were placed in universal tubes then centrifuged at 1000 *g* for 20 min. Plasma and thrombocytes were removed and the volume substituted by an equal amount of PBS added to the packed cell fraction. Sterile distilled water was added to lyse red blood cells. After 45 sec NaCl (2.7%) was incorporated to restore the isotonicity and the sample centrifuged at 2000 *g* for 20 min. Supernate was discarded and the pellet resuspended in HBSS then

centrifuged again (2nd wash) at 2000 *g* for 20 min. The pellet was resuspended in 1 ml of HBSS and the cells disrupted in a tissue grinder (2x for 5 min). The resulting ground cells were mixed with 70% SIP. 20 µl of green marker beads (density 1.102 g/ml) were added to the control tube and ultracentrifugation followed at 30000 *g* for 30 min. All material above the level of green beads was discarded. The remaining sample was washed 2x with HBSS at 1000 *g* for 20 min. Examination of Giemsa stained cytospins showed a good isolation of white blood cells (Fig 5.5). After 5 min of grinding there was some cell disruption but many of the cells appeared intact. After 10 min of grinding there was further disruption but too much froth was formed in the grinding process. Centrifugation in 70% SIP Percoll produced a good separation of cell debris that was above green density beads markers. It was expected that any *Ehrlichia* present should be in the area below the green beads (density 1.102 g/ml) (Woldehiwet *et al.*, 1991). The green marker beads were expected to be at 18 mm from the bottom of the tube.

The same procedure was used to purify antigens 11, 12 and 13 to be used in ELISA but with slight modifications. The sample was kept cold during the whole process, including ultracentrifugation that was performed at 4°C. Before grinding, 750 µl of proteinase inhibitor cocktail was added (7x stock solution, Boehringer Manheim). Grinding was performed at full speed for 15 min. Only one high speed wash (in HBSS 1x) at 10000 *g* for 20 min was performed. Green marker beads (density 1.102 g/ml) were at 18 mm as before. Just above the beads there was a faint white band. There were several thick bands of debris on top of the tube. It appeared clear underneath the green beads. Giemsa examination of cytospins showed that after 15 min grinding there was more cell disruption than before but still some neutrophils appeared intact (Fig 5.6). In the top fraction there was mainly cell debris. In the middle band there was cell debris plus some intact neutrophils and in the bottom there was some cell debris, some intact infected neutrophils and some free bodies that were presumably *Ehrlichia* (Fig 5.7).

Incubation of the samples after neutrophil isolation was used prior to grinding and ultracentrifugation because it has been found to promote multiplication of *Ehrlichia* morulae (Woldehiwet and Scott, 1982c) and the disruption of neutrophils.

For bulk isolation it appeared more efficient the use of preformed step-gradients, which allows the use of ordinary centrifugation tubes. Whole blood in heparin was collected and processed as before but under sterile conditions and using PBS for the washes instead of HBSS. Pellets containing the neutrophils were mixed with 10 ml of *Cowdria* medium (Appendix A) and incubated at 37°C, 5% CO_2 for 24-48 h. After incubation the samples were collected in universals and stored at -20°C without additives. Prior to use they were allowed to thaw then centrifuged at 15000 *g* for 20 min to pellet any free *Ehrlichia* present. The pellet was resuspend in HBSS (1x). Mechanical grinding was performed at full speed for 30 min keeping the sample on ice. 'Ballotini' glass beads grade 14 (Jencons Ltd, England) were included to help the disruption of the cells and prevent frothing. The resultant material was centrifuged at 15000 *g* for 20 min. The pellet was resuspended in HBSS and homogenised in a 10 ml tissue grinder. The sample was mixed with 70% SIP and the tubes were ultracentrifuged at 30000 *g* for 30 min or at 5000 *g* for 60 min, 4°C for preformed step-gradients (Fig 5.4.b). After centrifugation all cell debris was on top of the gradient above green marker beads as expected and the bottom appeared clear. Two fractions were taken, top and 1/3 of the bottom. The two different gradients were washed with HBSS 1x at 15000 *g* for 20 min, then the pellets resuspended in HBSS. Cytospins were made from top and bottom gradients, Giemsa stained and studied under light microscopy. After 48 h culture the sample looked more or less the same than after 24 h, some more *Ehrlichia* seemed to be free from the neutrophils (Fig 5.5). On the top gradient there was a lot of intact and disrupted cells and nuclei, some of them were neutrophils containing *Ehrlichia.* In the bottom gradient there were mainly cell debris and clumps of free *Ehrlichia* (Fig 5.7). The pellets were subjected to 2 high speed washes in PBS at 10000 *g* for 20 min at 4°C to eliminate Percoll. As much supernatant as possible was removed and the pellet resuspended by vortexing in 1 ml of PBS then it was transferred to 1.5 ml microcentrifuge tube and centrifuged at 13000 *g* for 10 min to pellet. The supernatant was removed and 1 ml of PBS was added then centrifuged at 13000 *g* to pellet and resuspended in 50 to 500 µl of PBS. Two cytospins were made after the final washes for both step and self-gradients. It appeared that the process of culture and freeze-thawing helped the cell disruption.

The bottom layers of self and step-gradients after washing contained clumps of *Ehrlichia* and less cell debris than prior to washing (Fig 5.7). Antigens 14, self and step were purified following the latest protocol.

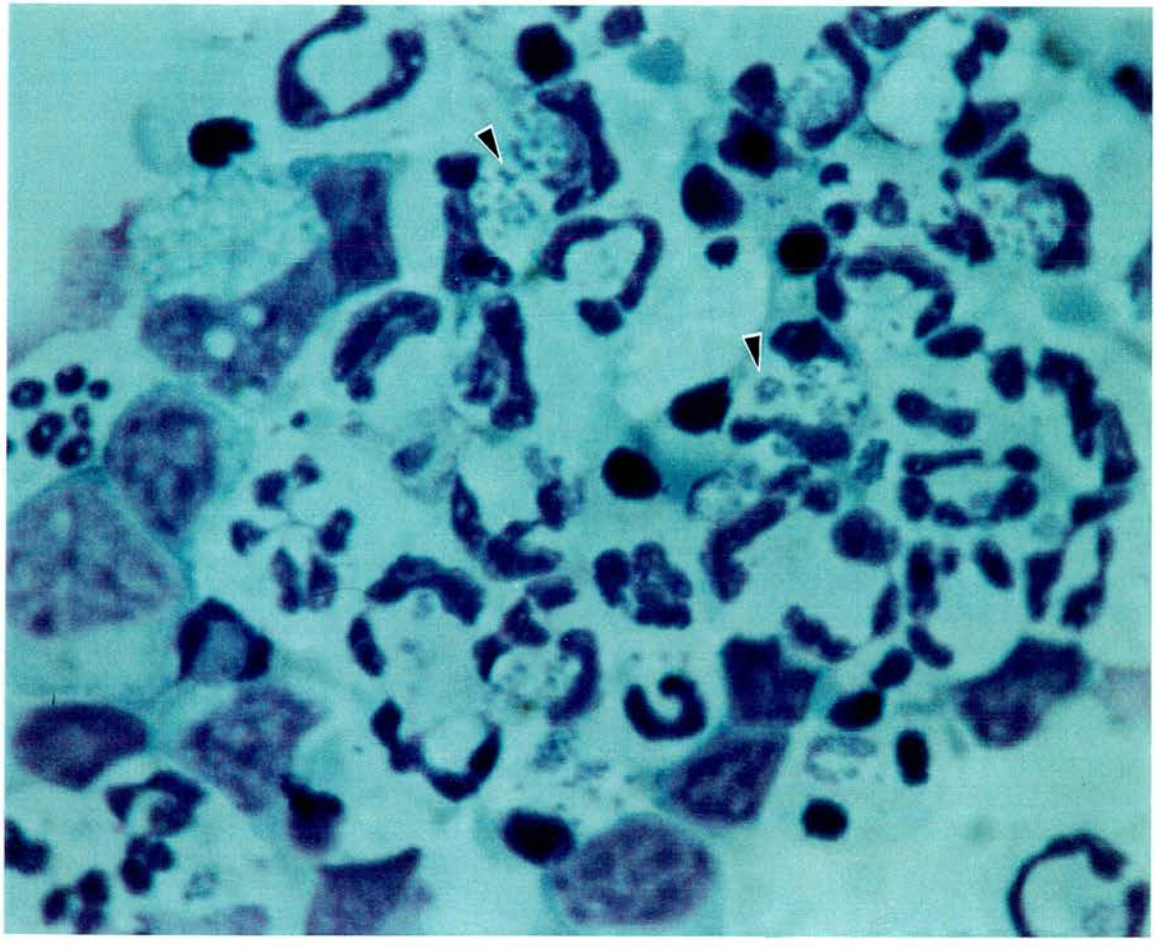


Fig 5.5 Giemsa stained cytocentrifuge showing a neutrophil preparation after 24 h culture at 37°C in *Cowdria* medium, before grinding. Overnight incubation has promoted neutrophil disruption and growth of morulae. Arrowheads indicate the presence of *E. phagocytophila* morulae (x1000)

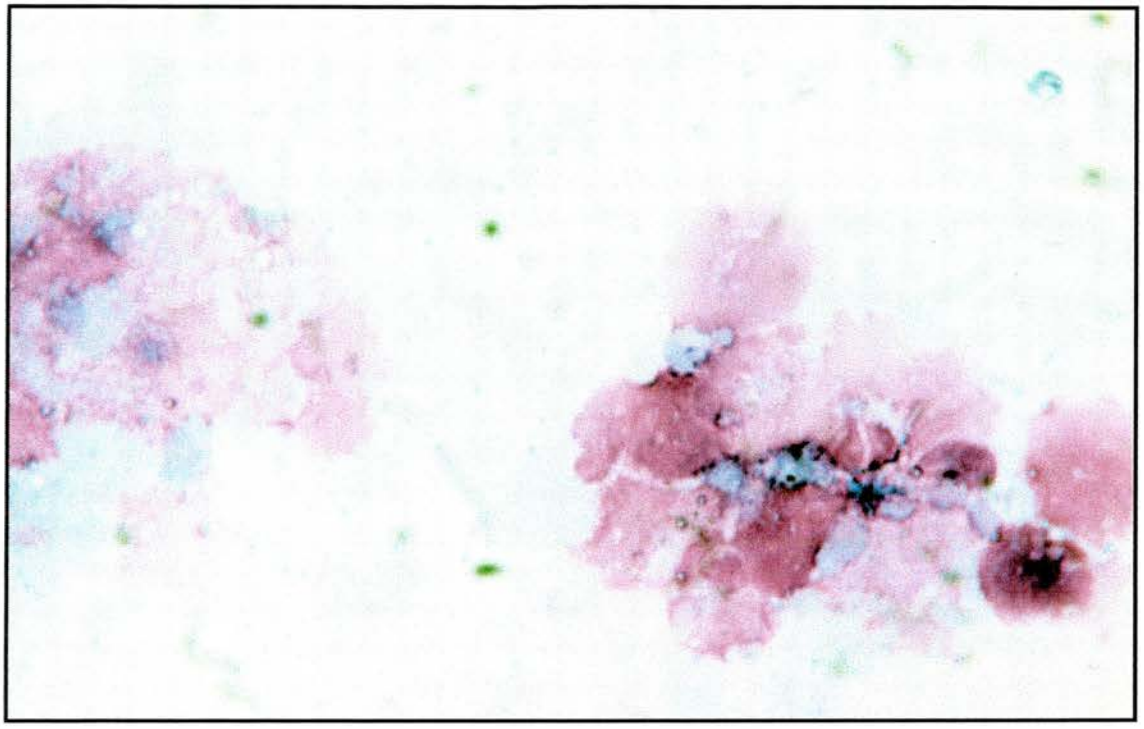


Fig 5.6 Giemsa stained cytocentrifuge showing a neutrophil preparation after grinding. Most cells appeared disrupted, *Ehrlichia* free bodies were difficult to identify (x1000)

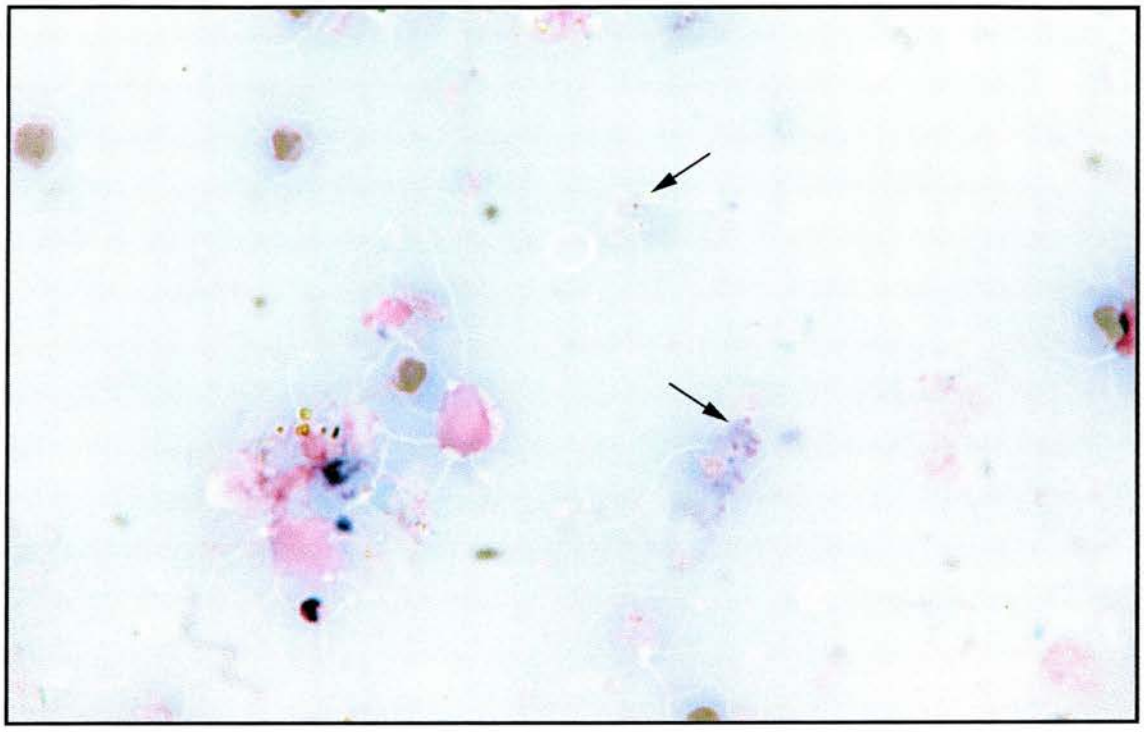


Fig 5.7 Giemsa stain bottom layer after Percoll centrifugation containing free *Ehrlichia* (arrows). Cell debris was still present even after several washes in PBS. (x1000)

5.2.2.12.4 Amplification by PCR of *E. phagocytophila* DNA in several Percoll purified fractions

PCR was performed using specific primers for granulocytic *Ehrlichia* to amplify a fragment of 410-bp from the *groE* gene following the conditions described in Chapter Seven. Four bottom fractions (pellets) corresponding to the antigens 11, 12, 13 and 14 used in ELISA were tested by PCR. Positive and negative controls were also included using DNA from sheep experimentally inoculated with *E. phagocytophila* and sterile distilled water respectively.

5.2.2.12.5 Competitive ELISA using monoclonal antibodies to *E. equi* and indirect ELISA with sheep/deer/cattle samples and *E. phagocytophila* as antigen

Competitive and indirect ELISA were performed as described in previous sections but using purified *E. phagocytophila* as antigen. Several dilutions in carbonate-bicarbonate buffer of antigen purified in six different occasions (antigens 11, 12, 13, 14, self and step) were used ranging from 1/50 to 1/500. Serum was diluted 1/200 in blocking buffer (PBST/2% normal rabbit serum). The species-specific HRP conjugate (Sigma) dilutions ranged from 1/8000 to 1/20000 in PBST.

5.2.3 Results

5.2.3.1 Peptide ELISA

In the preliminary peptide ELISA only reactions >5% were considered significant (Fig 5.8). Both samples with antibodies to *E. phagocytophila* and *E. canis* reacted to peptide 13. Interestingly, *E. canis* positive sera reacted more strongly to peptide 13HGE than to its homologue in *Cowdria* (13Cow). Peptide 61 (the four peptides with homologue amino acid sequence for the aoHGE, *E. chaffeensis*, *Cowdria* and *E. canis*) was recognised by samples containing antibodies to *E. phagocytophila* and *C. ruminantium*. Stronger responses were observed between homologues peptide-antiserum in the case of *Cowdria*. Peptide 82 showed more variation in the antibody reactions to the homologue peptides. 82HGE was

recognised by both sera with antibodies to *E. phagocytophila* and *Cowdria* but the reactions were stronger for *Cowdria* serum. 82cha was recognised by antibodies to *Cowdria* , *E. canis*, and a canine sample that was positive to *E. phagocytophila* by IFAT. 82Cow reacted with antibodies to *E. phagocytophila* and *Cowdria*. Peptide 89 (89HGE and 89Cow) gave the lowest reactions but it was selected for successive tests because it showed the biggest differences in the % of reactions in ELISA when the samples were tested with the presence-absence of the peptide indicating that much of the reactivity observed in the rest of the peptides was due to non-specific binding of antibodies or conjugate to the streptavidin. Sera containing antibodies to *E. phagocytophila* and *C. ruminantium* reacted to both 89HGE and 89Cow but the reactions appeared stronger with the homologous sera. Canine samples containing antibodies to *E. phagocytophila* as previously determined by IFAT did not show any difference in reaction when compared to negative serum (Fig 5.9).

In summary, the peptides used in this study did not appear to be specific enough to differentiate between rickettsial species. All peptides were equally definitive between positive and negative sera although higher reactions were obtained when using serum homologous to the peptide. It would be necessary to test a larger sample of known positive and negative species-specific serum to determine if these peptides could reliably be used on the field to pick up true positives avoiding cross-reactions between *Ehrlichia* species.

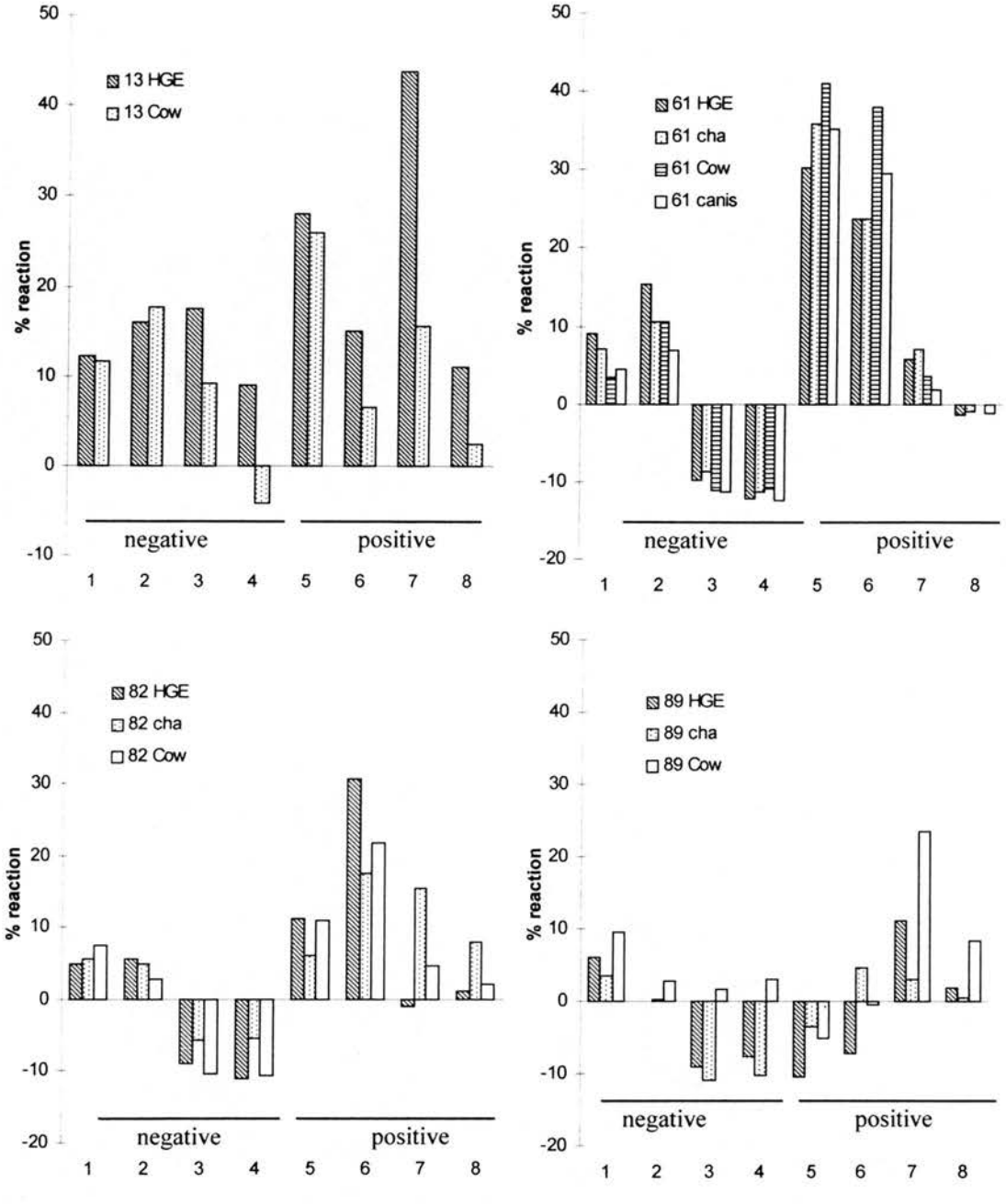
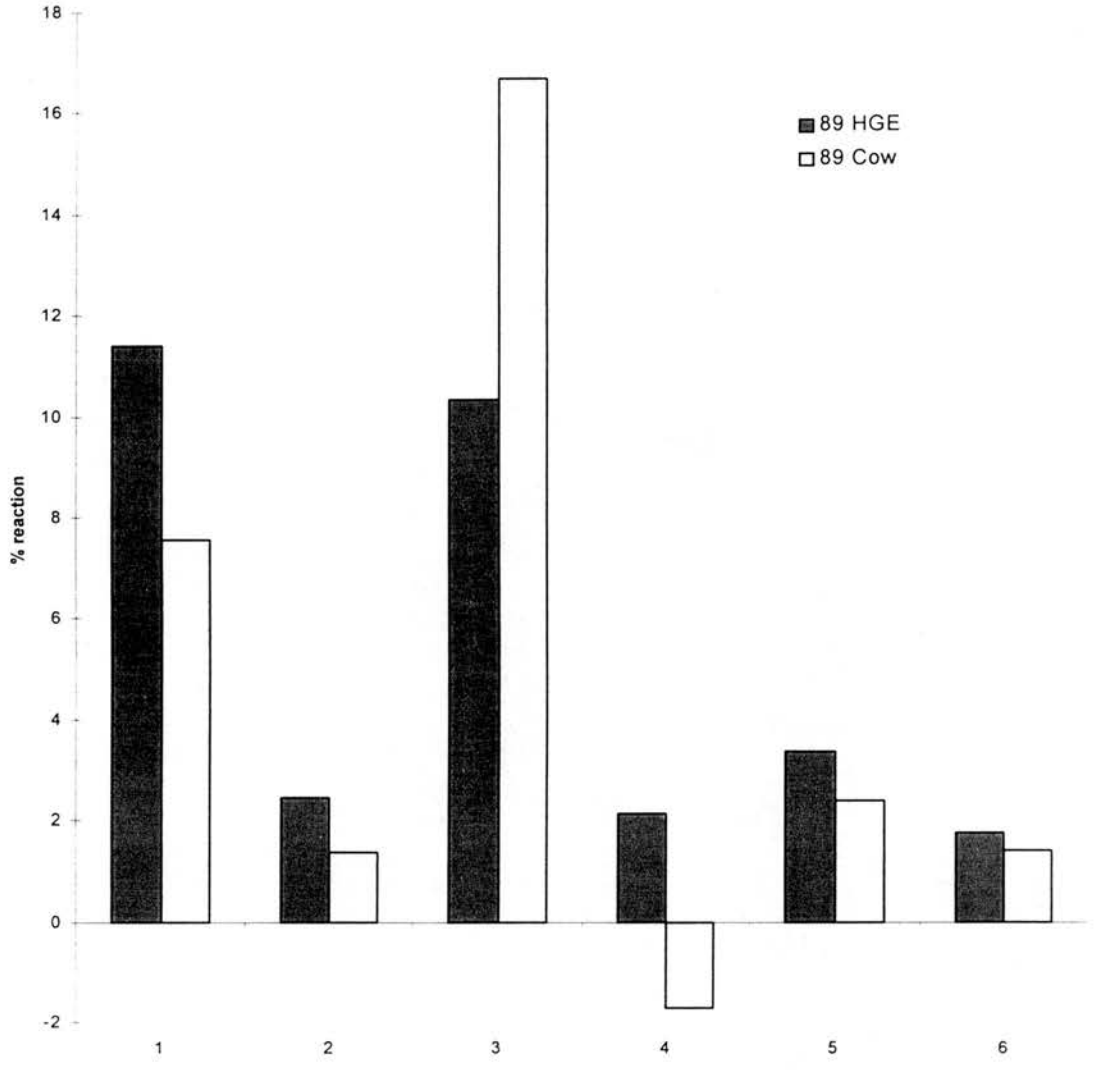


Fig 5.8 Preliminary peptide ELISA using 12 different peptides tested against negative and positive experimental sera raised to *E. phagocytophila* in sheep (samples 1 and 5), *C. ruminantium* (2 and 6), *E. canis* (3 and 7) and *E. phagocytophila* in dogs (4 and 8)



Positive to *E. phagocytophila*: 1, sheep; 5, dogs

Negative to *E. phagocytophila*: 2, sheep; 6 dogs

Cowdria ruminantium positive: 3; *C. ruminantium* negative: 4

Fig 5.9 Peptides 89HGE and 89Cow were tested against sera experimentally raised to *E. phagocytophila* and *C. ruminantium*. Four samples of canine origin that were previously identified as negative or positive to *E. phagocytophila* by IFAT were also included. Differences were found between positive and negative samples. Peptide 89HGE was recognised by both sera with anti-*Cowdria* and anti-*E. phagocytophila* antibodies. Peptide 89Cow seemed to be more specific for sera containing antibodies to *C. ruminantium*. Samples of canine origin with antibodies to *E. phagocytophila* reacted at very low level to both peptides

5.2.3.2 Monoclonal antibodies

Monoclonal antibodies titres ranged from <1/40 to 1/80 as determined by IFAT when using *E. phagocytophila* as antigen (Table 5.4). Only one, R5A9, reacted against *E. equi* in ELISA and at a low titre (Fig 5.3.a)

The monoclonal antibodies (MAbs) did not react to *E. canis*, *E. risticii*, *E. platys*, *Rickettsia rickettsii*, or *R. prowazekii* and they appeared to bind to a 44 kDa antigen in Western blot (Dr. D. Ravyn, personal communication). There seemed to be different recognition between the monoclonal antibodies since the ratio HGE-2/ *E. equi* varied from 16 to 2 fold (Table 5.4).

5.2.3.3 Indirect and competitive ELISA with tick-cell culture *E. equi* as antigen

5.2.3.3.1 Optimised competitive ELISA using BIg

Biotinylated IgG was titrated against *E. equi* 1/2000 diluted in carbonate-bicarbonate buffer (Fig 5.10.a). Best results were obtained in the range of 1/80 to 1/640 dilutions of BIg. The test was optimised using 1/160 dilution of BIg, 50% of serum and 1/2000 dilution of antigen (Fig 5.10.b).

The same samples derived from sheep experimentally inoculated with *E. phagocytophila* were tested by cELISA using BIg and MAb as competitors. The results are shown in Fig 5.11. The MAb seemed to discriminate better between known negative and positive samples.

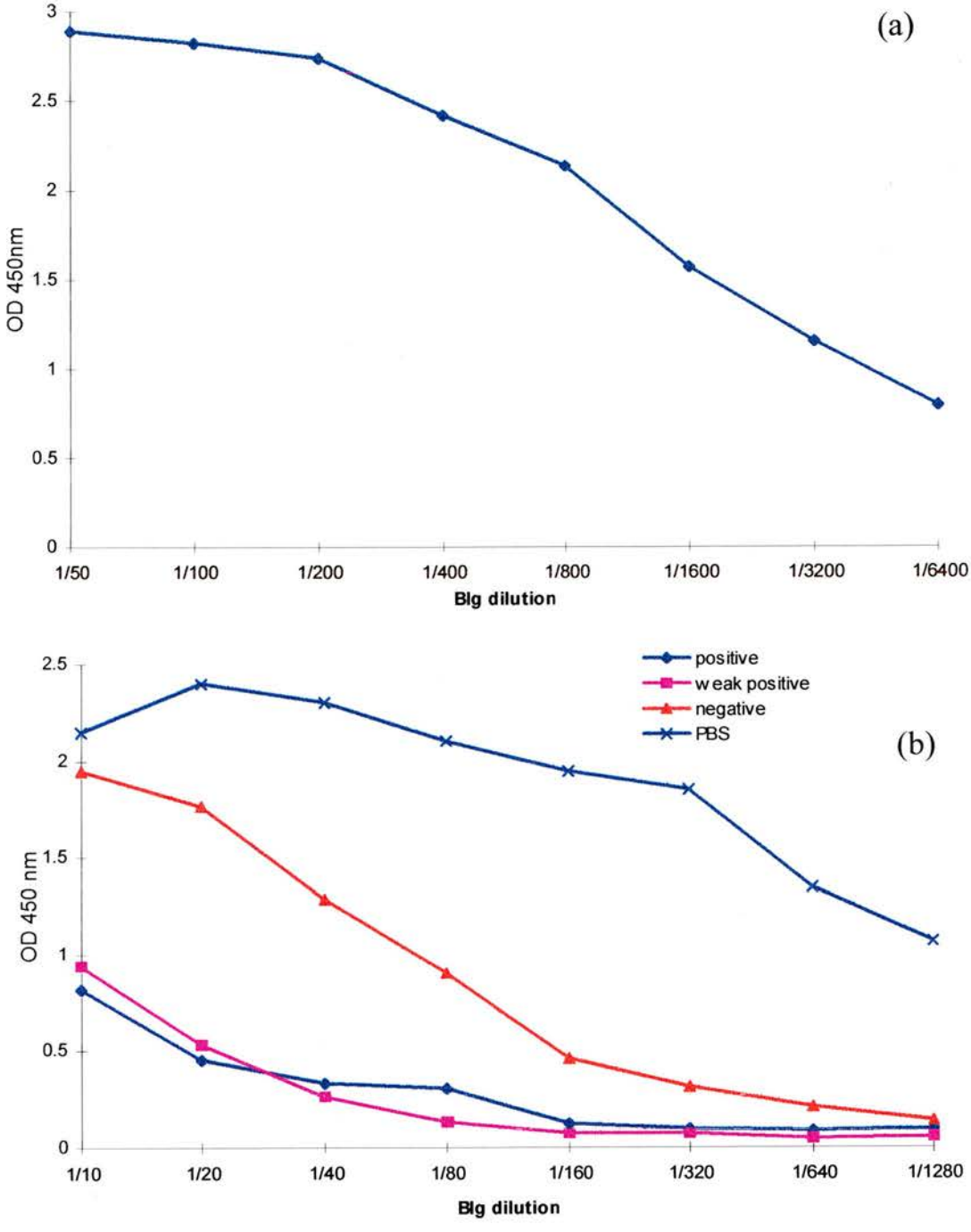


Fig 5.10 (a) BIg titration vs. *E. equi* antigen (1/2000 dilution); (b) BIg titration vs. anti-*E. phagocytophila* antiserum. Sample dilution was 50% for positive sera (with high and low titre) and negative sera

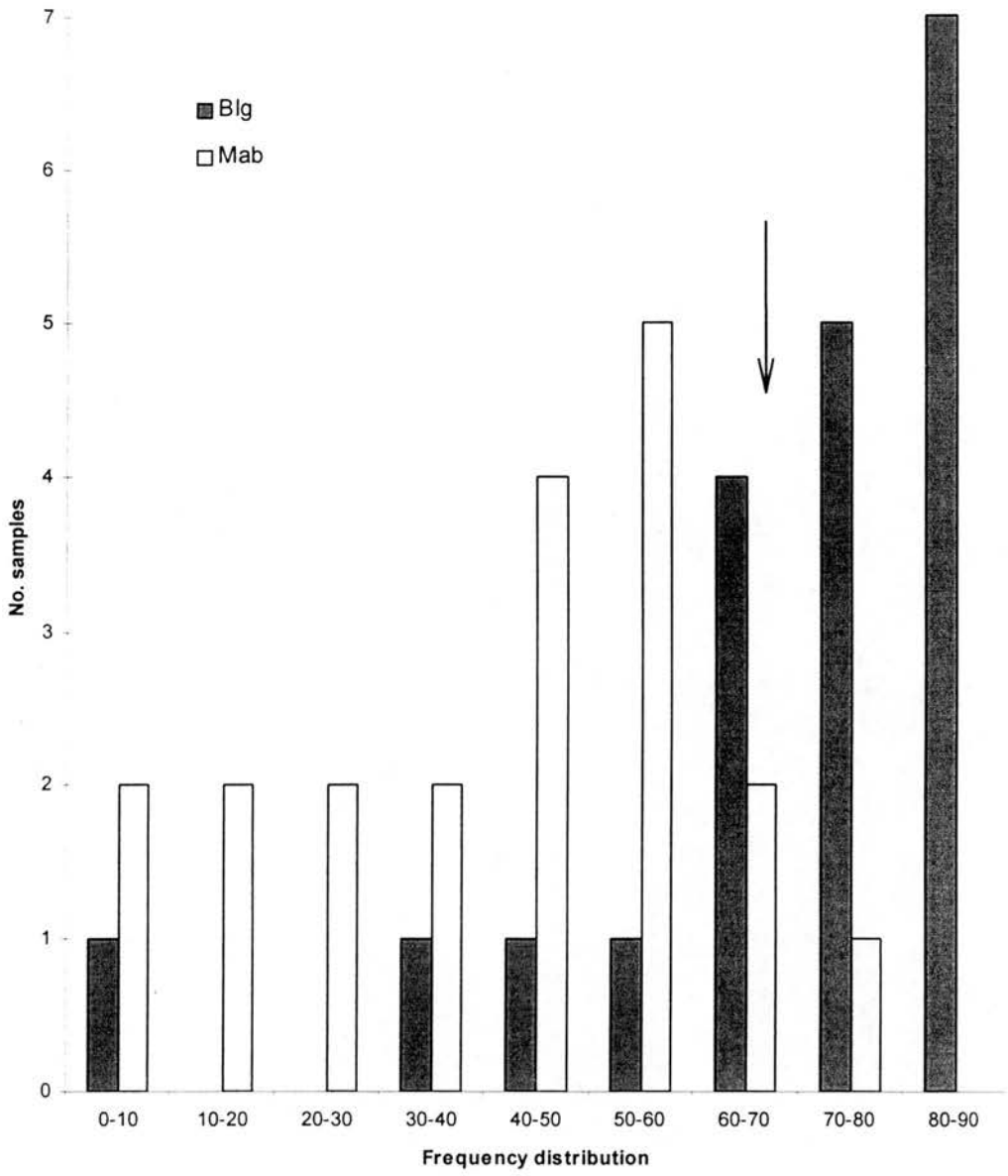


Fig 5.11 Comparison of results using BIg and MAb with the same experimental sheep sera. More known negative samples appeared to be positive when using BIg compared to MAb for the same cut-off point of >65% (arrow)

5.2.3.3.2 Optimised competitive ELISA using monoclonal antibodies

MAb R5A9 was selected to use in cELISA because it was the only one reacting to *E. equi* antigen (Fig 5.3.a). Antigen dilution was optimised at 1/8000 in carbonate-bicarbonate buffer. The best results were obtained when using 50% sera and 1/4 dilution of R5A9 MAb (Fig 5.3.b). MAb was added immediately after the serum at a dilution of 1/2 in PBST (final dilution 1/4). Background reactions of positive sera were reduced by increasing Goat anti-mouse horseradish peroxidase conjugate (Sigma) dilution to 1/20000 in PBST. Fig 5.12 shows a comparison of results between IFAT and cELISA at different times after experimental inoculation of sheep with *E. phagocytophila*. Previous IFAT results were confirmed by cELISA. A cut-off point of 50 % was selected for sheep species. Sera were negative at week one after experimental inoculation. Antibody levels started rising at week 2 and persisted after week 21 (sheep 936 and 937). The serum was able to compete with the MAb even at low titres (as determined by IFAT) indicating a high sensitivity for cELISA. Fig 5.13 shows a good discrimination between positive and negative serum from experimental sheep before and after inoculation with *E. phagocytophila*. Fig 5.14 indicates that the same cut-off points can be applied for roe deer samples. Sensitivity and specificity for both tests using experimental sheep serum is shown in Table 5.6.

Table 5.6 Competitive and indirect ELISA results compared to IFAT using experimental sheep serum containing antibodies to *E. phagocytophila*. Values are expressed as percentages

	cELISA	iELISA
Sensitivity	96	100
Specificity	100	90
Positive predictive value	100	91.67
Negative predictive value	93.75	100

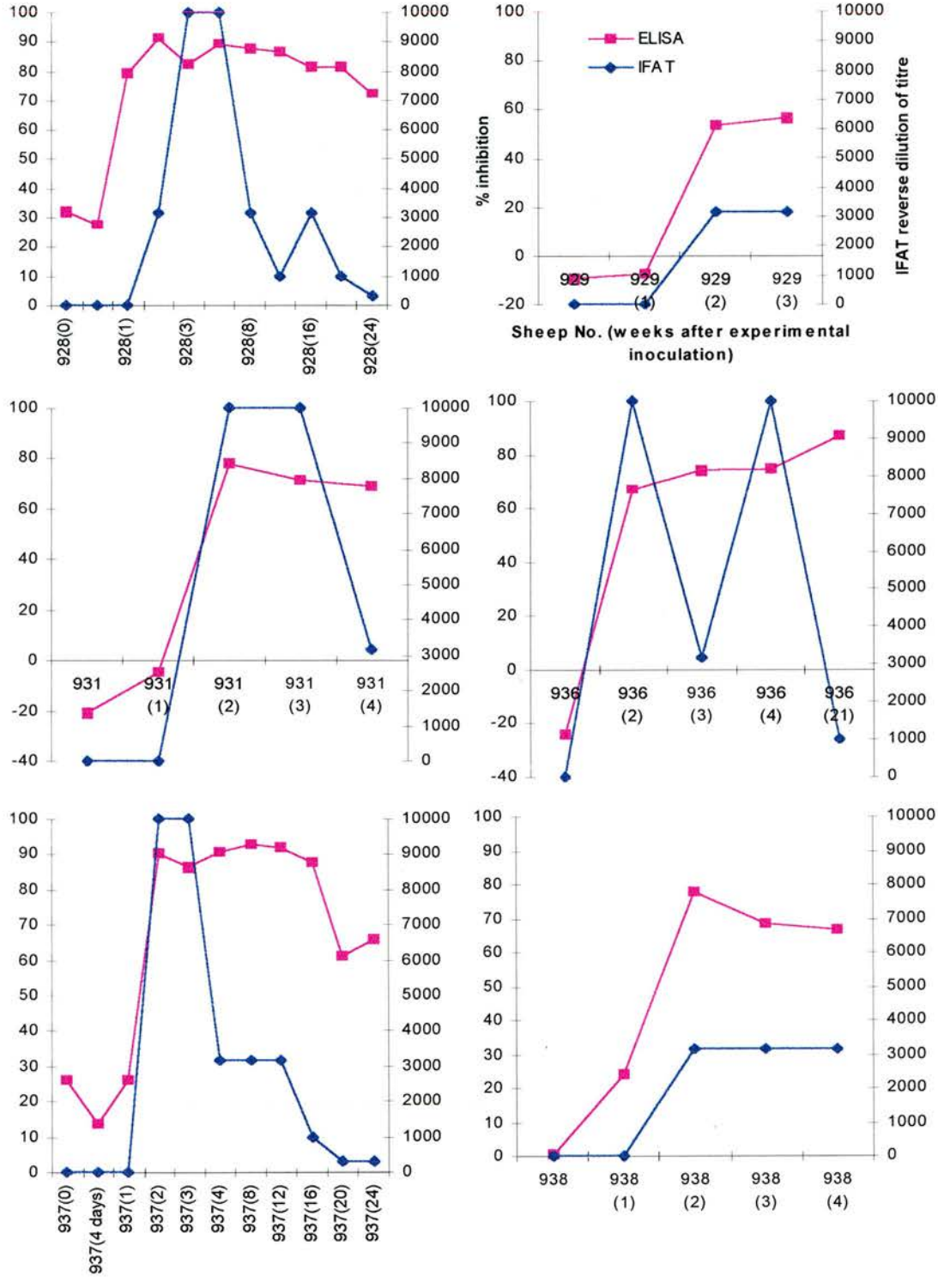


Fig 5.12 Comparison of results by IFAT and cELISA using six experimental sheep sera before and at different times after inoculation with *E. phagocytophila*

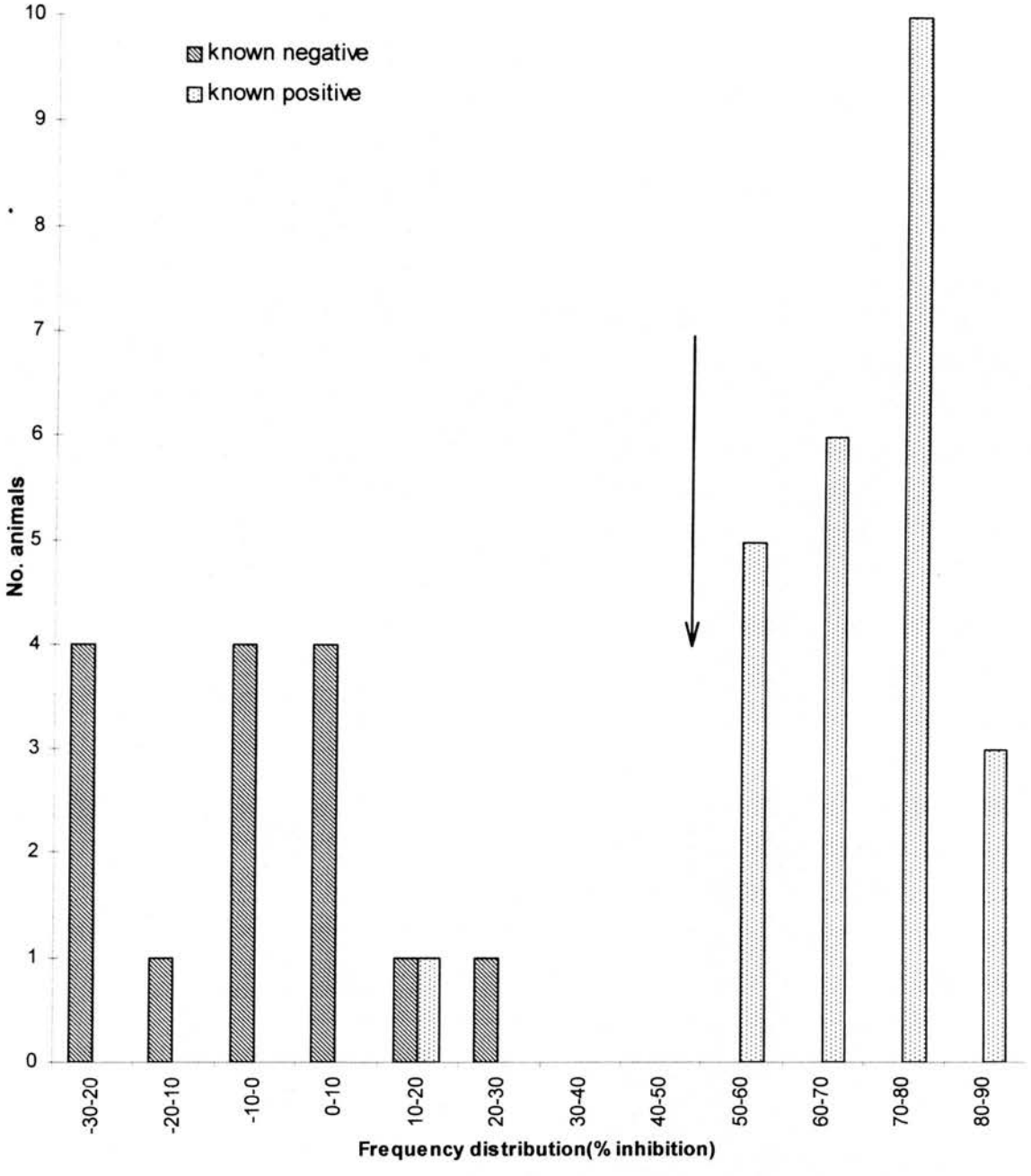


Fig 5.13 cELISA with experimental sheep sera before and after inoculation with *E. phagocytophila* using *E. equi* as antigen and monoclonal antibody R5A9. There was a good discrimination between samples previously determined by IFAT to be positive or negative. One sample was positive by IFAT at low titre two weeks after experimental inoculation and negative by cELISA using a cut-off point of >50% inhibition

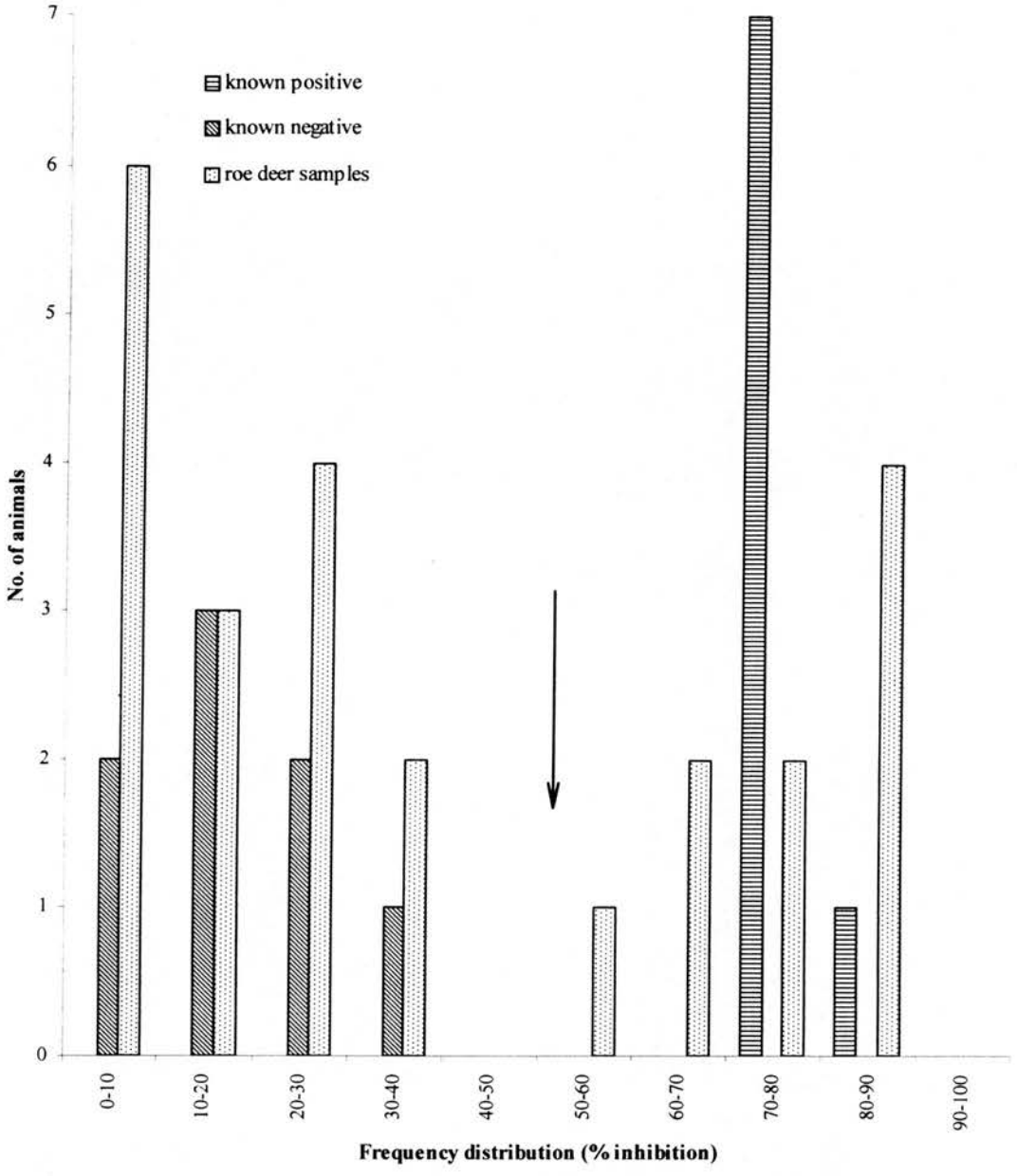


Fig 5.14 cELISA using *E. equi* as antigen and monoclonal antibody R5A9. Experimental sheep sera that were previously determined by as positive or negative by IFAT were compared to several field collected roe deer samples. A cut-off point of >50% appears adequate for both sheep and roe deer samples (arrow)

5.2.3.3.3 Optimised indirect ELISA

The chosen dilution for *E. equi* antigen in carbonate/bicarbonate buffer was 1/8000. Plates were blocked for 1 h in PBST/ 2% normal rabbit serum (blocking buffer) prior to incubation with sera. Serum was also diluted in blocking buffer and the plates incubated for 2 h. Donkey anti-sheep horseradish peroxidase conjugate (Sigma) was diluted 1/20000 in PBST. When compared to cELISA and IFAT (Table 5.6), iELISA appeared to have high sensitivity and specificity. Samples from the same experimental animals were used in the three tests.

iELISA results with sera from animals with and without antibodies to *E. phagocytophila* were compared using infected and non-infected tick cells. The observed reactions in positive antiserum appeared to be specific to *E. equi* and not to the tick components (Fig 5.15).

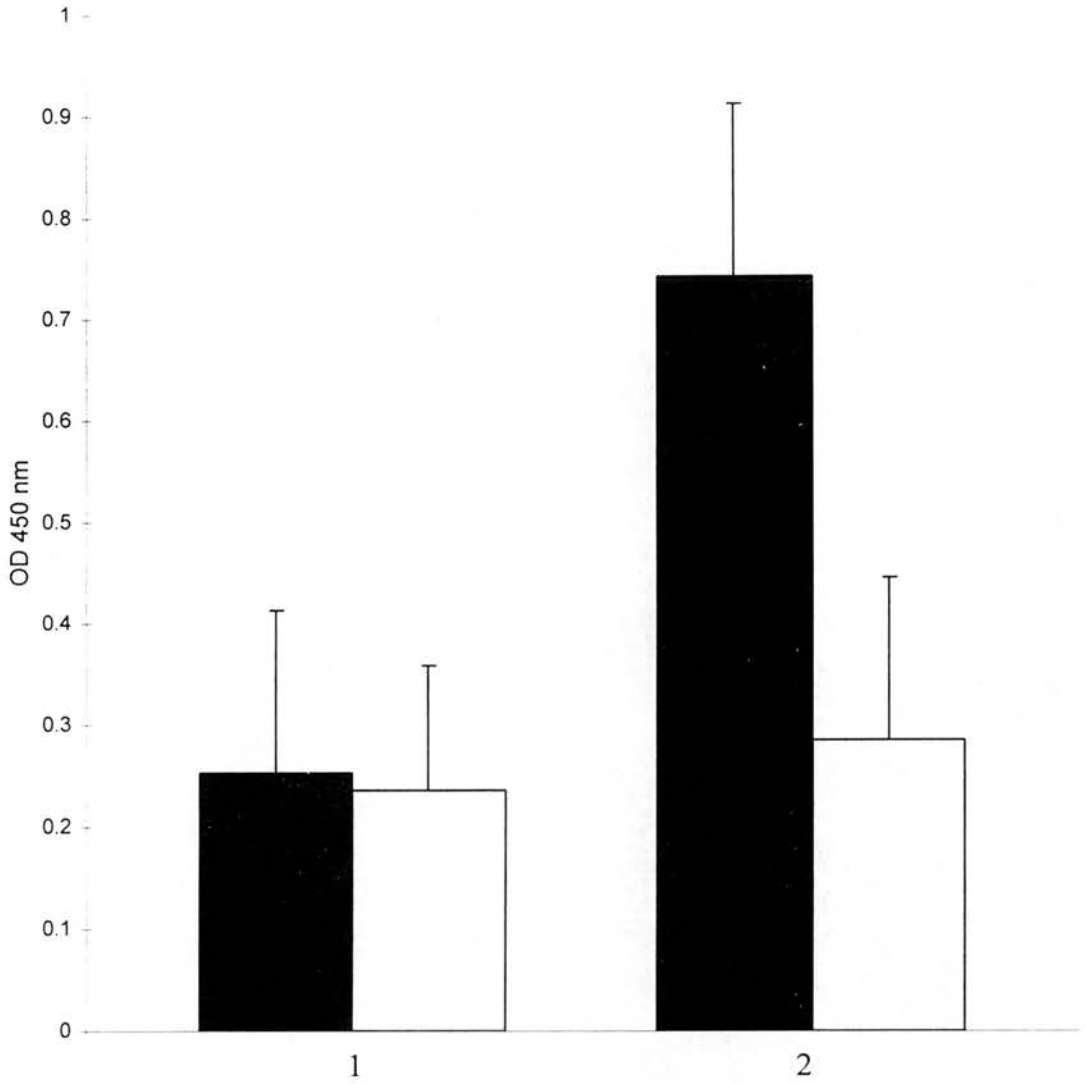


Fig 5.15 iELISA using *E. equi* infected (hatched columns) and non-infected (dotted columns) tick cells. (1) Mean negative sera; (2) Mean positive sera. Y error bars represent the standard deviations from the mean

5.2.3.3.4 ELISA results in different animal populations

After test optimisation, sera or plasma from different animal populations with no exposure to ticks or *E. phagocytophila* and samples derived from roe deer were tested and the results compared to previous results by IFAT (Table 5.7). Table 5.8 shows the results after competitive and indirect ELISA for roe deer samples collected in five different sites (Chapter Three) compared to IFA as a reference test. A comparison of IFAT, ELISA and Western blot results for 14 roe deer samples collected from different sites is shown in Table 5.9. Samples with low IFA titres did not appear positive by western blot. One of the samples that was identified as positive by ELISA and negative by IFA recognised the 44 kDa band characteristic for granulocytic *Ehrlichia* by western blot thus suggesting a higher sensitivity for ELISA compared to IFA. The rest of roe deer samples which were positive by ELISA or IFA or both were confirmed as truly containing antibodies to an *E. phagocytophila*-like pathogen.

ELISA frequency distributions for deer (Moredun institute), roe deer, dog and cat populations are shown in Fig. 5.16, 5.17 and 5.18.

Feline samples were derived from a population of cats that tested positive or negative to FIV. The presence of antibodies to the virus did not appear to interfere with the test results (χ^2= 0.09, p>0.05).

ELISA showed in general moderate agreement (0.41-0.6) with IFAT except for dog samples in which just slight agreement (0-0.20) between cELISA and IFA was observed (Table 5.7). Kappa coefficient arbitrary benchmarks are based on Everitt (1989). Very few canine positive samples were recognised by cELISA despite the high sensitivity observed for other species. This result is probably associated with the low titre of antibodies found in dog samples thus cELISA was unable to pick-up positive sera. On the other hand, it is possible that the strain of *E. phagocytophila* pathogenic for dogs presents a higher divergence than the rest from the *E. equi* antigen which was used in the study and was more similar to *E. phagocytophila* thus explaining the difference in the results.

Table 5.7 Comparison of serological results (expressed as %) of two different ELISA with the IFA reference test

Species	Roe deer		Moredun deer		Cats	Dogs
Type of test	iELISA	cELISA	iELISA	cELISA	cELISA	cELISA
(No. animals)	(86)	(52)	(60)	(60)	(51)	(90)
Se	81.25	89.66	47.62	76.19	90.91	17.65
Sp	76.32	65.22	92.31	79.49	80.00	95.89
PPV	81.25	76.47	76.92	66.67	55.56	50.00
NPV	76.32	83.33	76.60	86.11	96.97	83.33
P	56.98	55.77	35.00	35.00	21.57	18.89
kappa coefficient	0.58	0.56	0.44	0.54	0.58	0.18
observed agreement	0.79	0.79	0.77	0.78	0.82	0.81
chance expected agreement	0.51	0.52	0.59	0.53	0.58	0.77
SE	0.11	0.14	0.12	0.13	0.13	0.09
Z	5.34	4.13	3.58	4.20	4.36	2.02
p	<0.01	<0.01	<0.01	<0.01	<0.01	<0.05

Se	sensitivity	NPV	negative predictive value
Sp	specificity	P	prevalence
PPV	positive predictive value	SE	standard error of kappa

Table 5.8 Comparison of ELISA results (expressed as %) to the IFA reference test for roe deer samples from five different sites with low, medium and high seroprevalences (P) using inhibition >50 % and OD>0.45 as cut-off points for cELISA and iELISA respectively

		Borders	Kyloe	Auchtertyre	Euston	Moncreiffe	All sites
cELISA	Se	71.43	100	100	100	80	89.66
52 samples	Sp	83.33	0	0	0	0	65.22
	PPV	62.50	50	88	100	100	76.47
	NPV	88.24	0	0	0	0	83.33
	P	28	50	88.89	100	100	55.77
iELISA	Se	16.67	66.67	100	100	100	81.25
86 samples	Sp	100	28.57	0	0	0	76.32
	PPV	100	54.55	85.71	90	100	81.25
	NPV	83.87	40	0	0	0	76.32
	P	18.75	56.25	85.71	90	100	55.81

Table 5.9 Comparison of ELISA, IFAT and western immunoblot results for 14 roe deer samples which showed low and high antibody titre to *E. phagocytophila*

No.	Ref.	Site	c-ELISA	i-ELISA	IFAT	IFAT titre	Western blot
2	KU250295	Borders	+	-	+	1/400	-
7	KU041195	Borders	+	+	+	1/6400	+
91	KU211098	Borders	ND	ND	+	1/100	-
100	KU011198	Borders	ND	ND	+	1/100	-
25	CM150398	Kirkhouse	+	+	+	1/3200	+
27	AY170398	Auchtertyre	+	+	+	1/6400	+
68	AY270598	Auchtertyre	ND	+	+	1/12800	+
71	AY040698	Auchtertyre	ND	+	+	1/6400	+
89	AY061098	Auchtertyre	ND	ND	+	1/6400	+
37	JB280398	Kyloe	+	+	+	1/3200	+
41	JB110498	Kyloe	+	+	-	-	+
51	HR300498	Euston	+	+	+	1/12800	+
57	HR160598	Euston	ND	+	+	1/12800	+
78	HR170798	Moncreiffe	ND	+	+	1/3200	+

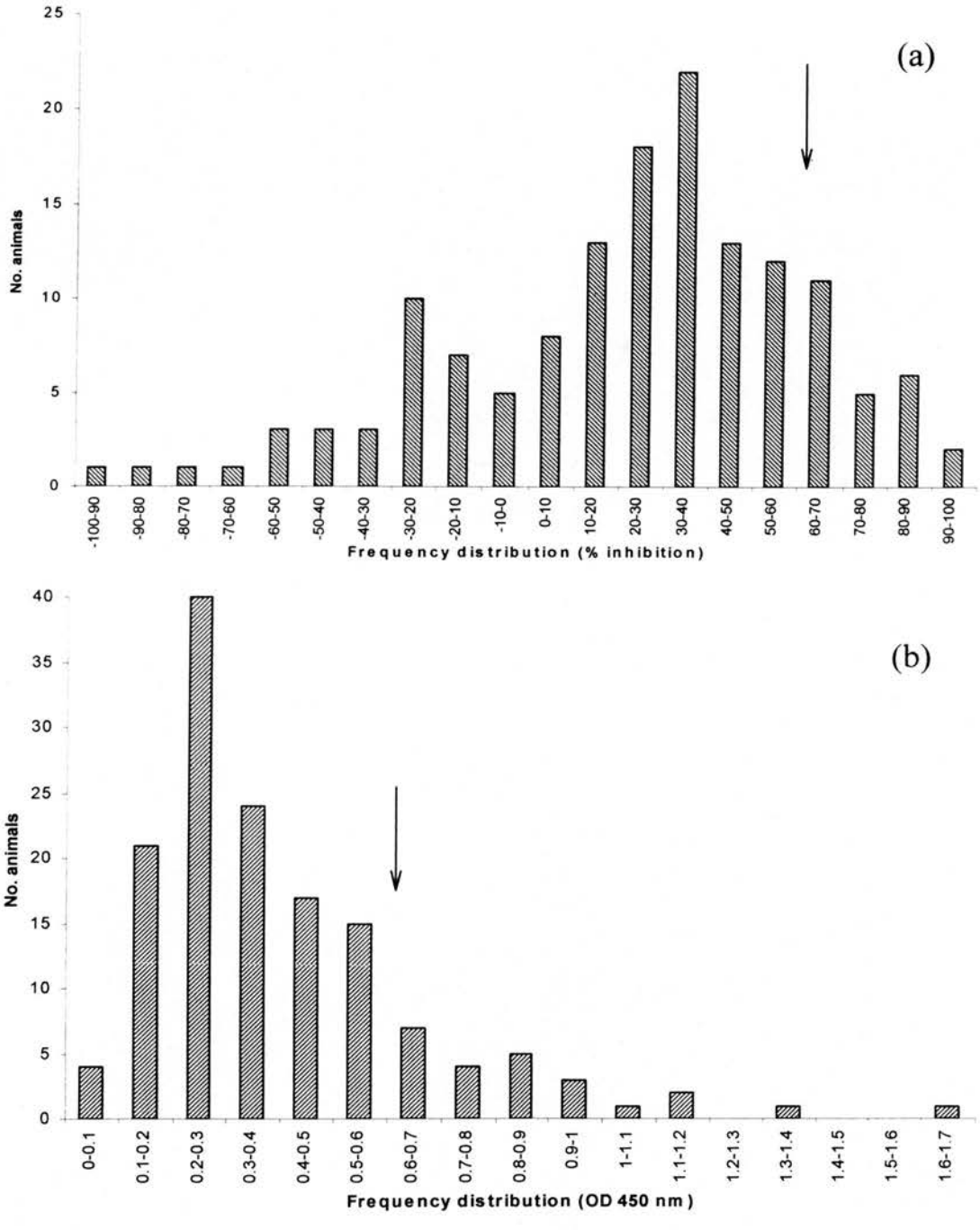


Fig 5.16 Frequency distribution of 145 deer samples obtained from the Moredun institute that tested negative to louping-ill virus. Cut-off points of inhibition >60% and OD >0.6 were respectively established for (a) cELISA and (b) iELISA. All intervals used to create the frequency distributions in this study were mutually exclusive

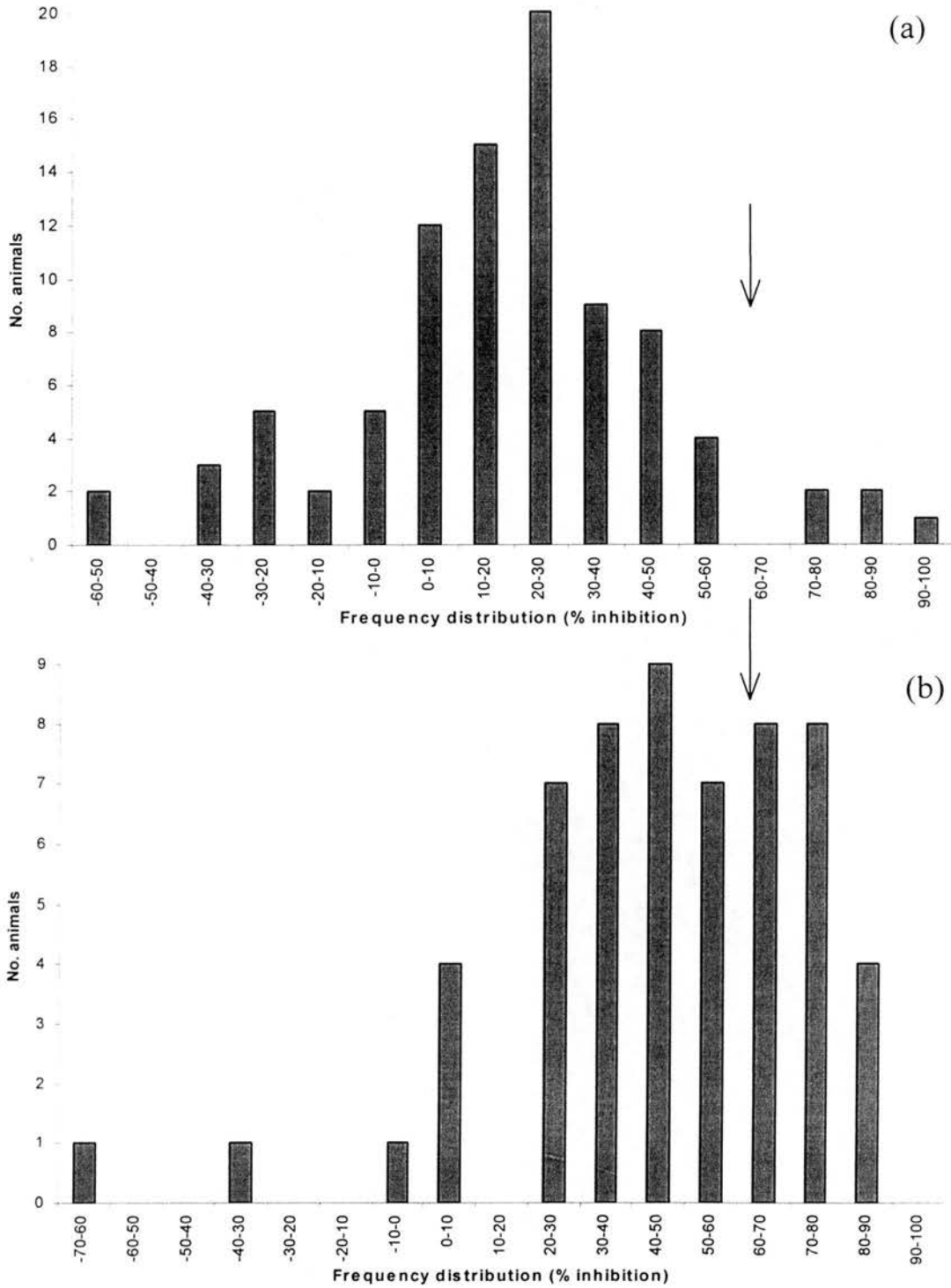


Fig 5.17 (a) Frequency distribution for 90 dogs that were tested previously by IFAT (b) Frequency distribution of 51 cat samples that were tested against FIV virus. A cut-off point of ≥ 60% was established for both populations

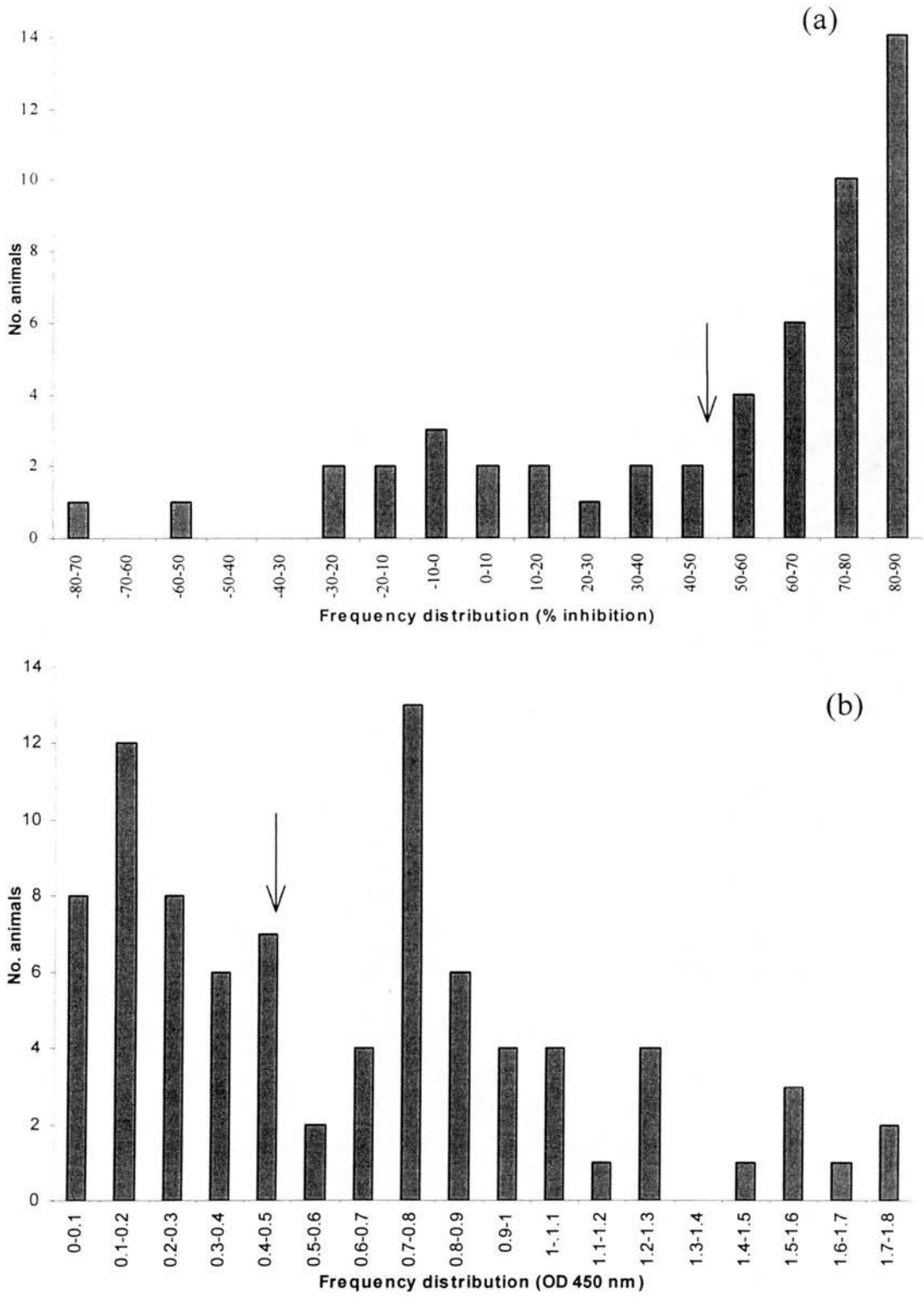


Fig 5.18 Frequency distribution of 52 roe deer samples after testing by (a) cELISA and (b) iELISA (86 samples). Cut-off points of inhibition >50% and OD>0.45 were respectively established

5.2.3.4 Percoll density gradient purified native *E. phagocytophila* antigen

5.2.3.4.1 Experimental inoculations of sheep

Infection was attained in all four sheep. They showed clinical signs characteristic of TBF infection and heir rectal temperatures were high from days 3 to 9. Giemsa staining of blood smears revealed infected neutrophils with typical morulae shaped bacteria inside. Table 5.10 summarises rectal temperatures and parasitaemia found in experimental sheep after inoculation with Ehr/8 isolate of *E. phagocytophila*.

Table 5.10 Results after experimental inoculations of sheep with *E. phagocytophila* for the isolation of native antigen

Day after	Sheep No., T (°C)				Sheep No., parasitaemia (%)				
inoc.	872	887	319	970	872	887	319	970	Other details
0					0	0	0	0	1 ml 1/10 Ehr/8 in PBS I/V inoc.
2		40.9				1			
3	41.5				10				
4		41.8				14			
5	41.1		41.8	41.8	10		22		
6	41.0	41.4	41.4	41.6	31	29	32	24	blood in heparin
7		41.5	41.7	39.4		26	42	55	blood in heparin
8	40.4	41.2	41.5	41.3		13	27	19	blood in heparin
9			40.8	39.6			18	10	blood in heparin
10	39.8								blood in heparin
20									plasma in heparin
34									plasma in heparin
35									plasma in heparin
49									plasma in heparin

5.2.3.4.2 Antigen isolation from infected neutrophils with Percoll density gradients

Several approaches were tried in order to isolate *Ehrlichia* from infected neutrophils as explained in section 5.2.2.12. After neutrophil disruption and Percoll centrifugation the pellet was washed one or two times at high speed in order to reduce the presence of contaminants in the sample. It was decided to use the following purified antigens labelled as 11, 12, 13, 14, 'self' and 'step'. The last three antigens were thoroughly washed twice at high speed in PBS.

5.2.3.4.3 PCR amplification of *E. phagocytophila* DNA in several Percoll purified fractions

E. phagocytophila DNA was amplified from all four antigens 11, 12, 13, and 14 indicating the presence of the bacteria in the bottom fractions after ultracentrifugation on Percoll density gradients (Fig 5.19).

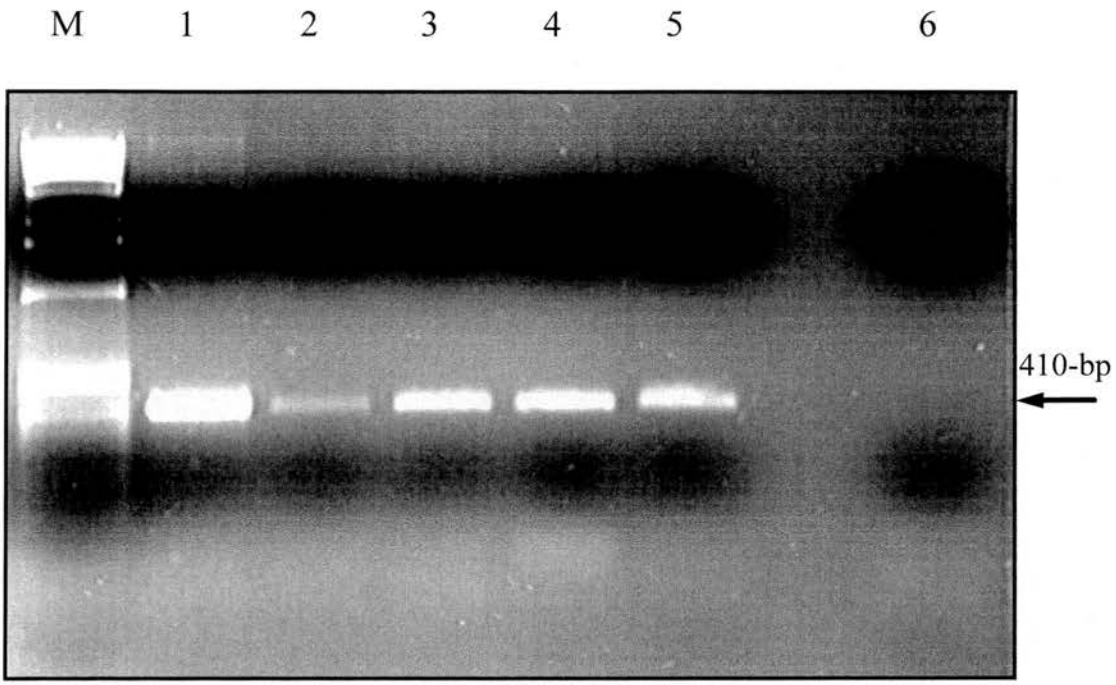


Fig 5.19 G*roEL* gene PCR using specific primers for granulocytic *Ehrlichia* to amplify a 410-bp fragment (arrow) from Percoll purified fractions containing *E. phagocytophila* DNA. M, molecular weight marker (1 Kb), Lane 1; *E. equi*; Lanes 2-5, fractions 11, 12, 13, and 14 separated after centrifugation in Percoll density gradients; Lane 6, sterile distilled water

5.2.3.4.4 cELISA trials using monoclonal antibodies to *E. equi* and iELISA with sheep/deer/cattle samples using *E. phagocytophila* as antigen

MAb R5A9 was titrated against *E. phagocytophila* as antigen in cELISA as previously described in section 5.2.2.11.2. However, no reactions were observed indicating that the monoclonal antibody was species specific for *E. equi.*

Several purified antigens were tested by iELISA at different dilutions and using sera from different species. Antigens 11, 12, and 13 were not as thoroughly washed as 14, self and step antigens. No difference was found between crude and processed antigen. Better results were obtained for antigens 14, self and step (1/50 dilutions in carbonate/bicarbonate buffer) but only for deer sera. Cattle and sheep sera did not show any difference when using negative and positive samples and sometimes negative sera gave even higher background reactions. It was suspected that the samples were cross-reacting against sheep components thus suggesting the antigen was not pure enough (Fig 5.20).

17 roe deer samples were tested by iELISA using *E. phagocytophila* crude Ag 14. Their results were compared to those previously obtained when using *E. phagocytophila* and *E. equi* in IFAT and iELISA respectively. The specificity of the iELISA was similar for both antigens but *E. equi* proved to be more sensitive to detect positive roe deer samples when compared to IFAT (Table 5.11).

Table 5.11 The same roe deer samples were tested using two different iELISA with *E. equi* and *E. phagocytophila* as antigens. Results (expressed as percentages) were compared to the ones obtained by IFAT using *E. phagocytophila* infected neutrophils

	Type of antigen	
	E .equi	*E. phagocytophila*
Sensitivity	100	62.50
Specificity	77.78	77.78
Positive predictive value	80	71.43
Negative predictive value	100	70
Seroprevalence	47.06	47.06

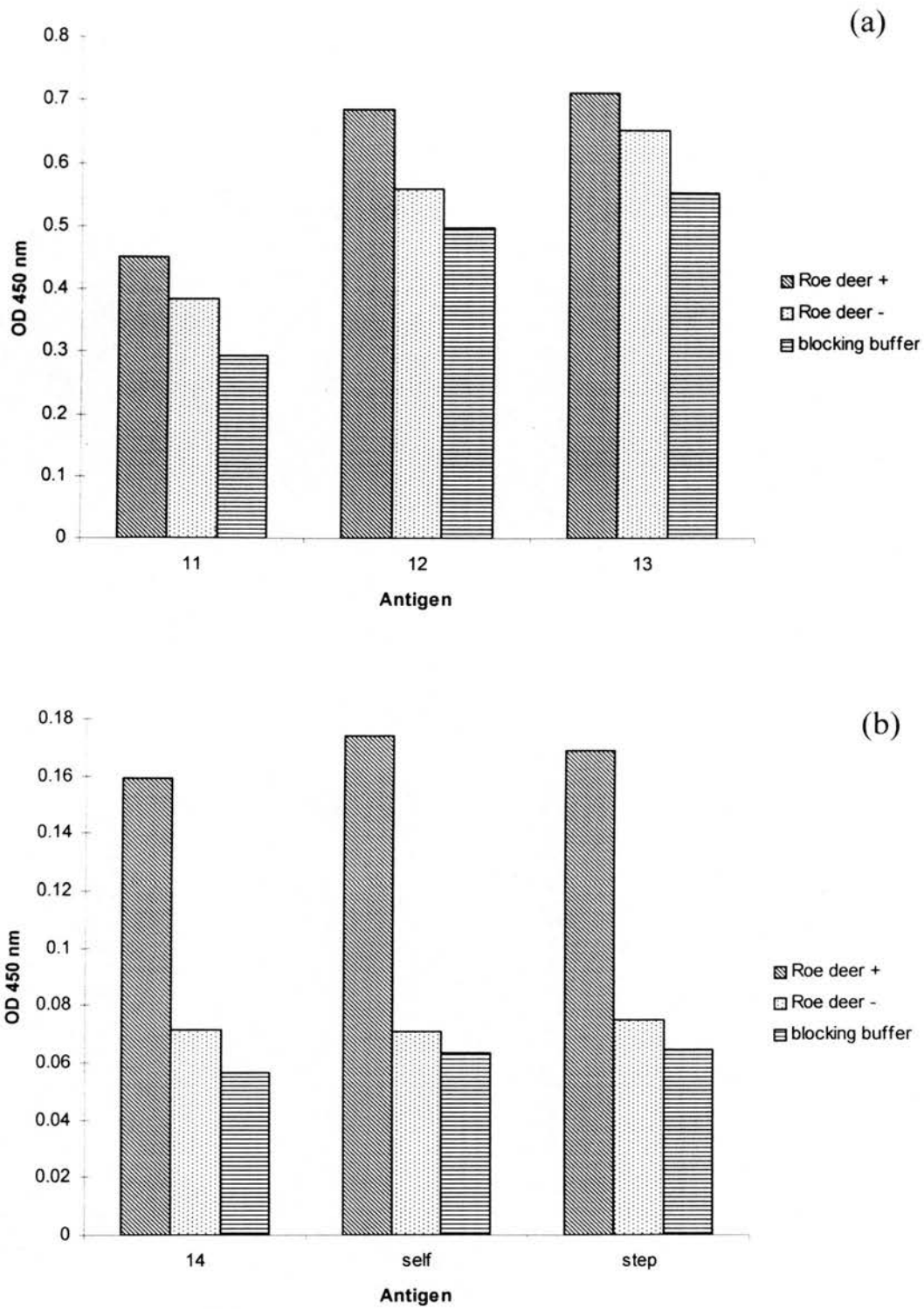


Fig 5.20 iELISA using *E. phagocytophila* antigens purified on Percoll density gradients. (a) Antigens 11, 12 and 13 (b) Antigens 14, self and step that were washed twice at high speed in PBS. Differences were found between roe deer positive and negative samples when using antigens 14, self and step

5.2.4 Discussion

It is not possible to cultivate *E. phagocytophila* in vitro, hence the main source of bacteria is infected sheep. This has hampered the development of serological tests for the diagnosis of the pathogen. IFA is routinely used for the detection of antibodies to *E. phagocytophila* (Paxton and Scott, 1989). It can also be used as a surrogate for *E. equi* and cases of granulocytic ehrlichiosis in different hosts under the evidence of serologic relationship and cross-reactions between granulocytic *Ehrlichia* (Dumler *et al.,* 1995). Despite IFA sensitivity and specificity, the assay is labour intensive and the interpretation of results subjective requiring skilled personnel to perform it.

In this study, peptide ELISA was developed in the hope that specific peptides would be identified for each of the *Ehrlichia* species used in ELISA. That would avoid the serologic cross-reactions between *Ehrlichia* and *Cowdria* species, a major drawback in the diagnosis and epidemiological studies of the diseases caused by those pathogens in places where both are endemic. Unfortunately no such peptides were identified. Samples containing specific antibodies to *Ehrlichia* and *Cowdria* species cross-reacted with peptides from the same region but of different bacterial species. The design of peptides was based on the *groEL* amino acid sequence deduced from the known nucleotide sequences deposited in GenBank database for each of the bacterial species used in this study as previously identified (Sumption *et al.*, 1997). Possibly, there was not enough amino acid variation among the peptides thus allowing cross-reactions between *Ehrlichia* species. More research is necessary in order to identify regions in the bacterial genome not so well conserved between species that will allow the design of specific peptides for diagnostic and epidemiological purposes. Kolbert *et al.* (1997), when using a recovered protein based on the HGE *groEL* homologue, managed to detect antibodies in mice experimentally inoculated with HGE whereas no reactions were seen in antiserum from mice infected with *Borrelia burgdorferi*. However no antiserum to other *Ehrlichia* species was investigated. Cross-reactions are likely to occur in *Ehrlichia* species when using proteins based on *groE* operon since it appears to be highly

conserved within eubacteria (Sumner *et al.*, 1993). The intergenic spacer between the open reading frames *groES* and *groEL* of the *groESL* (*groE*) operon appears more promising because it shows differences in length and nucleotides between *Ehrlichia* species (Sumner *et al.*, 1997).

Nyika *et al.* (1998) used the major antigenic protein gene (MAP1) of *Cowdria ruminantium* as a vaccine in laboratory mice, obtaining from 23 to 88% survival rates suggesting DNA vaccines may be protective against heartwater and other rickettsial diseases. However, this gene is encoded by a polymorphic multigen family (Sulsona *et al.*, 1999) inducing variable antigenic determinants by recombination which may explain the persistence of carrier infections in animals. On the other hand, the use of DNA regions encoding major immunodominant single proteins as vaccines may not induce the desirable protective responses against the pathogen variants found in nature. Variation in the size of two surface antigens from different strains of the causative agent of Potomac horse fever, *E. risticii,* has been observed (Biswas *et al.*, 1998). Vaccine failures against the bacteria may be associated with the observed heterogeneity between isolates.

Differences in antigenicity between granulocytic *Ehrlichia* isolates are known to occur (Zhi *et al.*, 1997; Ravyn *et al.*, 1998). The three isolates used in this study for serology (*E. equi*, *E. phagocytophila* Feral Goat, and *E. phagocytophila* Ehr/8) may also differ in major antigenic epitopes thus leading to the diverse although consistent serologic results obtained. iELISA, although less sensitive than cELISA, appears to be the assay of choice in situations with a high prevalence of infection because it gives a reduced number of false positive results. In contrast, cELISA is particularly useful in areas where a low prevalence of infection is expected due to its higher sensitivity. Both tests need to be optimised further for non-ruminant species with samples derived from known negative populations of animals. Although cELISA proved to be very specific for canine samples, it was not able to detect serum that was previously identified as positive by IFAT. However, all titres obtained for canine samples were very low (see Chapter Three) and false positive results by IFAT may also occur. When using known sheep negative and positive samples both competitive and indirect ELISA were highly sensitive (96%) and specific (100%)

suggesting that the comparison of ELISA results with IFAT as the 'gold' standard test for other species of unknown status may not be ideal because of the possibility of obtaining false positive or negative results. Unfortunately, no known negative populations for other species without tick and/or *Ehrlichia* exposure were available to further validate the assays.

Percoll density gradients have been used for the isolation of several *Ehrlichia* species (Weiss *et al.*, 1989; Rikihisa *et al.*, 1995) and *Cowdria ruminantium* (Neitz *et al.*, 1986). The objective of this study was to isolate *E. phagocytophila* free from the cell components to use as a crude antigen in ELISA. The addition of trypsin to help digestion and disruption of the cells has been recommended for the isolation of *E. risticii* by some authors (Rikihisa *et al.*, 1994) although the aim of their purification was different. Cell disruption was however the main problem encountered in this part of the experiment but trypsin was avoided because it has been found to destroy antigenic epitopes in *E. chaffeensis* (Chen *et al.*, 1996). In some studies they have managed to isolate *Ehrlichia* to use as antigen for immunoblots using 30% diatrizoate meglumine density gradients after sonication of infected leucocytes, however the yield of antigen did not appear to be high enough (Dumler *et al.*, 1995).

High buoyant densities are characteristic of *Ehrlichia*. Dutta *et al.* (1987) purified *E. risticii* to use in ELISA using Renografin gradients at a density of 1.182 g/ml. Woldehiwet *et al.* (1991) purified *E. phagocytophila* from ovine blood by Percoll and Renografin density gradients, its buoyant density ranging from 1.063 to 1.040 g/ml. They also encountered difficulties to isolate the bacteria free from any cell component. For this study all the material above 1.102 g/ml was discarded to make sure only free *Ehrlichia* was harvested. The use of plasma instead of whole blood appears to be a good source of *E. phagocytophila* free from cellular contamination when examined by electron microscopy (Woldehiwet *et al.*, 1991). However, because other rickettsial species can be present free in the plasma such as *Eperythrozoon ovis*, a pathogen that infects erythrocytes (Neitz *et al.*, 1934; McKee *et al.*, 1973), for antigenic studies *E. phagocytophila* should be isolated from infected neutrophils, which are less likely to contain *E. ovis*.

Ehrlichia phagocytophila crude antigen obtained in this study was not pure enough to use in routine ELISA. The main problem appeared to be attaining full disruption of neutrophils to set the bacteria free from cell components because a sonicator was not available at the time. Thus cell contaminants were found in the pellet containing free *Ehrlichia* even after several washes in PBS. In addition, some other high density particles such as mitochondria could also be present in the bottom fractions. The individuals are known to produce low levels of normally occurring antibodies to mitochondrial epitopes (NOMA) (Baum, 1995). That may explain the lack of differences in background reactions when using experimentally infected sheep sera prior and after inoculation with *E. phagocytophila*. In addition, false positive results have been recorded when bovine erythrocyte proteins contaminating *Anaplasma marginale* antigens were detected by anti-erythrocyte antibodies in bovine sera (Duzgun *et al.*, 1988). However, samples from roe deer that were previously identified as positive or negative by IFAT and ELISA, using *E. phagocytophila* and *E. equi* antigens respectively, showed consistent differences when using the purified *E. phagocytophila* antigen in iELISA. The results are promising and indicate that the use of Percoll gradients to isolate the bacteria combined with other techniques such as magnetic beads or chromatography columns for further purification might provide a standardised supply of antigen to use in ELISA until culture methods for the organism are successfully established as for *E. equi* (Munderloh *et al.*, 1996b) and the HGE agent (Goodman *et al.*, 1996). However, isolation by affinity chromatography appears to impair rickettsial infectivity as observed when inoculated into susceptible sheep (Woldehiwet *et al.*, 1991) and may affect, perhaps, its antigenic properties in ELISA. Monoclonal antibodies raised to *E. equi* did not appear to recognise *E. phagocytophila* antigens. Despite the antigenic and genetic relationship between granulocytic species which allows the use of surrogate antigens for diagnostic purposes, differences in titre and epitope recognition were found thus confirming the need for specific tests for each of the strains or bacterial species. In addition, although two closely related organisms may share similar antigens, they may not stimulate equally strong antibody responses in the animals.

It appears possible to recover a potential antigen from the blood of an animal infected with *E. phagocytophila* without prior biological amplification in an animal model or culture. Kolbert *et al.* (1997) expressed a portion of a fragment from the *groEL* ORF amplified by PCR and used the fusion protein as antigen to determine its immunoreactivity in a murine model of HGE infection. Knowles *et al.* (1996) developed a highly specific cELISA using a recombinant protein epitope of major surface protein 5 (MSP5) for *A. marginale* as antigen. However, targets easily amplified by PCR in different bacterial species are also more likely to produce immunodominant antigens and therefore show cross-reactivity. The identification of less conserved genes or even the construction of genomic libraries appears desirable for this uncultured organism in order to identify specific antigens for serology and for comparative studies between granulocytic strains of *E. phagocytophila.*

CHAPTER SIX, ANTI-MITOCHONDRIAL ANTIBODY RESPONSES INDUCED BY *RICKETTSIAL* INFECTIONS

6.1 Introduction

Mitochondria organelles are believed to have evolved from eubacteria-like endosymbionts. They present a residual genome subject to a high mutation rate and they are abundant in most cells. Their function in the eukaryotic host cells is to help in the energy conservation reactions. The closest known relatives are the rickettsial group of α-subdivision of the purple bacteria (α-Proteobacteria) that includes the animal pathogens *Ehrlichia phagocytophila* and *Cowdria ruminantium* as demonstrated at molecular level by sequence analysis of 16S ribosomal DNA (Weisburg *et al.*, 1991) and Hsp60 (heat-shock 60 proteins, chaperonins, or *groEL* in bacteria) (Viale and Arakaki, 1994) suggesting they shared the last common ancestor. In addition, mitochondria and the *Ehrlichia/Rickettsia* cluster share the characteristic of being intracellular endosymbionts or obligate parasites of eukaryotic cells, including the ability to escape the phagolysosome action and reproduce inside eukaryotic cells. Hsp60 are highly conserved proteins very adequate for the inference of the origin of organelles since they are present in both eubacteria and eukaryotic cells.

The immune system would be expected to be tolerant to self-peptides of mitochondrial origin because during development all T-cell clones with receptors recognising mitochondrial peptides would have been eliminated or anergised. In addition, mitochondria are isolated from humoral responses by their intracellular location. However, the individuals are known to produce low levels of antibodies to them (Naturally Occurring Mitochondrial Antibodies, NOMA) (Baum and Berg, 1981; Baum, 1995). On certain occasions the levels of antimitochondrial antibodies are very high as in autoimmune diseases triggered by recurrent bacterial infections. It is suspected that phylogenetically closely related pathogenic bacteria mimic mitochondrial epitopes. That similarity exacerbates the normally occurring autoimmune response to mitochondria, particularly after repeated exposure. Some tissues are known to be especially targeted like the liver or kidneys, in which mitochondria are abundant.

Primary biliary cirrhosis (PBC) is a chronic liver disease associated with a higher incidence of recurrent urinary tract infection. It is the commonest reason for having a liver transplant in Europe (Baum, 1995). The damage to the liver is believed to be associated with a continuous bacterial exposure triggering an autoimmune response, 95% of the patients showing high levels of autoantibodies against mitochondria (Baum, 1995). It is commonly associated with autoimmune diseases such as rheumatoid arthritis or thyroiditis but it is considered to be an autoimmune disease in itself (Mackay and Gershwin, 1989) inducing a multi-system disorder.

Another example is rheumatic carditis, which is known to be a sequel of streptococcal infection. Naturally occurring antibodies to heart components are usually present but their levels are increased after *Streptococcus mutans* exposure. Autoimmune mechanisms have been suggested for this process as well. There is evidence that the heart component myosin cross-reacts with anti-streptococcal monoclonal antibodies (Kohler and LoVerde, 1988). Group A streptococci may activate autoantibody producing B cell clones. Also, *S. mutans* has shared epitopes with cardiac muscle and it is known to cause subacute bacterial endocarditis (Kohler and LoVerde, 1988). Bacterial components may stimulate self-reactive B-lymphocytes leading to autoantibody production or *S. mutans* itself may damage the cardiac muscle, then hidden self-antigens may be exposed inducing autoimmune responses.

The most common mitochondrial antigen, M2, in patients with primary biliary cirrhosis is a family of 4 major antigens in beef heart mitochondria with approximate molecular weights of 74, 56, 52, and 48 kDa (Butler *et al.,* 1993). It is known that M2 is loosely associated with the inner surface of the inner membrane of mitochondria. The 74 kDa antigen cross-reacts with bacterial membrane fractions (Butler *et al.,* 1993). There is little evidence showing that some anti-*Ehrlichia* responses might be anti-mitochondrial. Anti-M2 anti-mitochondrial autoantibodies (AMA) have been found to react against several bacteria (Baum, 1995) not so closely related phylogenetically as mitochondria are known to be to the *Ehrlichia/Rickettsia* cluster (Butler *et al.,* 1993; Viale and Arakaki, 1994). Thus, the existence of a high

level of anti-mitochondrial antibodies after rickettsial infection would not be surprising.

Ehrlichia phagocytophila infections are known to induce a weak but prolonged antibody response. Humoral immune responses may not be fully protective against the organisms since they are intracellular pathogens, although high levels of antibodies correlate positively with resistance to reinfection (Woldehiwet and Scott, 1982a). Host protective immune responses are expected to be directed against surface antigens of invading pathogenic organisms. Major antigenic outer membrane proteins are usually involved in the interaction between intracellular pathogens and host cells. The close similarity of mitochondrial and ehrlichial antigenic epitopes may influence the antibody response towards the pathogen. In this case, tolerance to self-peptides needs to be balanced with immuno-competence against phylogenetically closely related bacteria.

Thrombocytopenia is a pathognomonic feature of *E. canis* infection. It appears to be associated with a platelet migration inhibition factor (Kakoma *et al.*, 1978) similar to the one found in human autoimmune disease (Duquesnoy *et al.*, 1975). In addition, it has been suggested that the hypergammaglobulinemia observed does not correlate with protection but may even enhance the pathogenesis of ehrlichiosis (Burghen *et al.*, 1971) and is related to a state of autoimmunity induced by the infection.

The aim of this study was to determine if the antibody response mounted after exposure to the rickettsial pathogens *Ehrlichia phagocytophila* and *Cowdria ruminantium* recognised beef heart mitochondrial proteins. The identification of antigenic epitopes shared by both mitochondria and *Ehrlichia* may help our understanding in the nature of the humoral immune response towards rickettsial pathogens which may be essential in order to develop effective vaccines against them.

6.2 Materials and Methods

6.2.1 Serum samples from sheep and goats

Sheep and goat sera containing specific antibodies to *E. phagocytophila* and *C. ruminantium* after experimental inoculations were used, kindly provided by Edith Paxton. Sera from sheep three weeks after challenge with the same strain of *E. phagocytophila* (Feral Goat) were also included in the study. Pre-exposure sera to either infection from the same animals was available as controls to detect any anti-mitochondrial activity prior to inoculation. Sera were aliquoted and kept frozen at -20°C until used. Foetal calf serum was used as a control for SDS-PAGE western blotting and ATPase assays.

6.2.2 Preparation of mitochondrial antigens for electrophoresis

Preparations from bovine heart mitochondria (protein concentration 20 mg/ml) were kindly donated by Dr. David Apps (Biochemistry Department, University of Edinburgh). Samples were stored at -20°C. Before use they were allowed to thaw at room temperature then diluted in sample buffer (Appendix B) to a final concentration of 5 mg/ml of protein. Diluted samples were boiled for 5 min, and 100 µl aliquots loaded into polyacrylamide gels (500µg of protein per gel). 10µl of pre-stained or biotinylated broad range molecular size markers (BioRad) were also loaded to obtain an estimate of the protein sizes.

6.2.3 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), western immunoblotting and staining of mitochondrial antigens

Slab gel electrophoresis was carried out as described by Laemmli (1970) at 200 volts for 45 min using discontinuous polyacrylamide gels (4-10%, Appendix B). Proteins were transferred (Trans-blot® SD Semi-dry Transfer Cell, BioRad) to nitro-cellulose sheets (Hybond) for 30 min at 10 volts, and 0.52 amperes. After blocking overnight in 5% blocking buffer (Appendix B) and washes in PBST, immunoblots were incubated with sheep sera (1/50 dilutions in blocking buffer) for an hour. After further washes in PBST, a secondary antibody, donkey anti-sheep IgG peroxidase conjugate (Sigma) was added (1/500 dilution in blocking buffer), and incubated

shaking for further 60 min. After further washes in PBST, immunoblots were developed with 4-chloro-1-naphthol diluted in cold methanol with the addition of H_2O_2 diluted in TBS. Blots were incubated with the solution until the bands acquired the desired intensity. The reaction was stopped by washing in sterile distilled water.

6.2.4 BN-PAGE and denaturing second dimension Tricine-SDS-PAGE

Blue native polyacrylamide gel electrophoresis (BN-PAGE) isolate respiratory chain proteins as complexes, which are visible as distinct bands thus allowing the identification of proteins rather than just their molecular weights. Further resolution of proteins in the complexes were achieved by second dimension tricine SDS-PAGE to separate proteins of less than 100 kDa (Gels in this section were kindly performed by Dr. David Apps from the Biochemistry Department in the University of Edinburgh).

6.2.5 Spectophotometric assay of ATPase activity by phosphate release

The aim of this experiment was to determine if antibodies to *E. phagocytophila* inhibited mitochondrial ATPase activity, the main enzymatic complex (Complex V) to which anti-mitochondrial activity appeared to be directed (Dr. Apps, personal communication).

Normal mitochondrial ATPase activity was measured by testing several concentrations of the purified mitochondrial proteins following protocols of Lebel *et al.* (1978). 200µl of assay mix were aliquoted into 5ml tubes and warmed to 30°C. 50µl of the diluted membrane (from 0 to 100 nmol per tube) were added to start the reaction, then incubated to 30°C for 10 min. 900µl of reagent A (0.34 M sodium acetate, 2 M acetic acid, 0.25% $CuSO_4.5H_2O$) were added to stop the reaction followed by 150µl of reagents B (5% ammonium molybdate) and C (2% p-methylaminophenol sulphate, 5% Na_2SO_3). The samples were incubated again for 10 min at 30°C. Their absorbance was read immediately at 850 nm (UNICAM 8625 UV/VIS spectrometer). As a blank the same mitochondrial dilution was used for each tube but the reaction was stopped by adding immediately (without incubation) reagent A, followed by reagents B and C. A standard curve was used to calculate the

amount of phosphate released from ATP due to the mitochondrial ATPase activity (see Results, Fig 6.4). Reagents A, B, C and ATP assay mix components are listed in Appendix B.

The effect in the assays of the presence of serum containing antibodies was tested by using two pre-infection and two post-infection sera after inoculation with *E. phagocytophila*. As a negative control two foetal calf sera were included, in which no background reactivity was expected.

6.3 Results

6.3.1 SDS-PAGE gels and Western blot analysis of mitochondrial antigens

Immunoblots showed activity towards mitochondrial proteins in samples containing antibodies to *E. phagocytophila*. Most of the samples recognised two distinct bands of approximately 48 and 70 kDa. Some pre-exposure samples reacted weakly towards a 48 kDa protein (Fig 6.1). Goat sera containing antibodies to *C. ruminantium* showed a similar pattern, post-exposure samples reacted mainly to 48, 52 and 58 kDa bands (Fig 6.2). Pre and post-challenge sera, both containing antibodies to *E. phagocytophila,* also recognised the main 48, 52, 58 and 70 kDa bands (Fig 6.3).

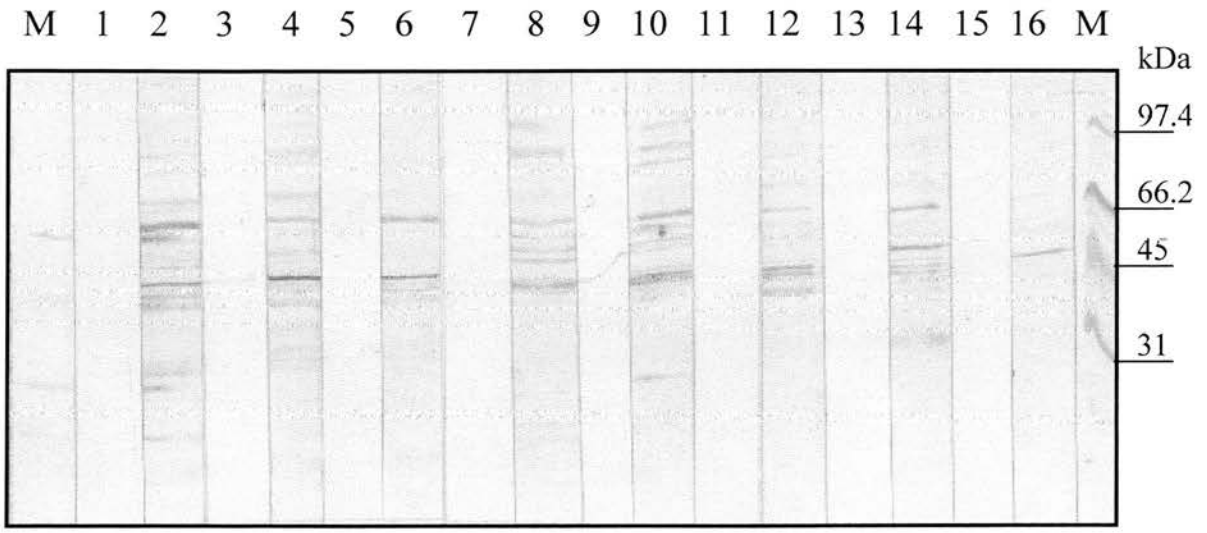


Fig 6.1 Mitochondrial antigens identified in immunoblots by sheep antiserum. Mitochondrial proteins were separated by SDS-PAGE, transferred to nitro-cellulose sheets and western blotted. M, molecular size markers; Odd lanes, pre-exposure sheep sera; Even lanes, post-exposure sheep sera after experimental inoculation with *E. phagocytophila*. Arrows indicate the approximate molecular size in kDa of the resolved proteins. A 48 kDa protein was recognised by all post exposure sera and also two pre-exposure sera (lanes 3 and 9) although the reactions were weaker. No more bands were observable in pre-exposure samples. In contrast, a strong antibody response was elicited against mitochondria after exposure. A protein of approx. 70 kDa was common to all infected sera, many weaker bands were also observed

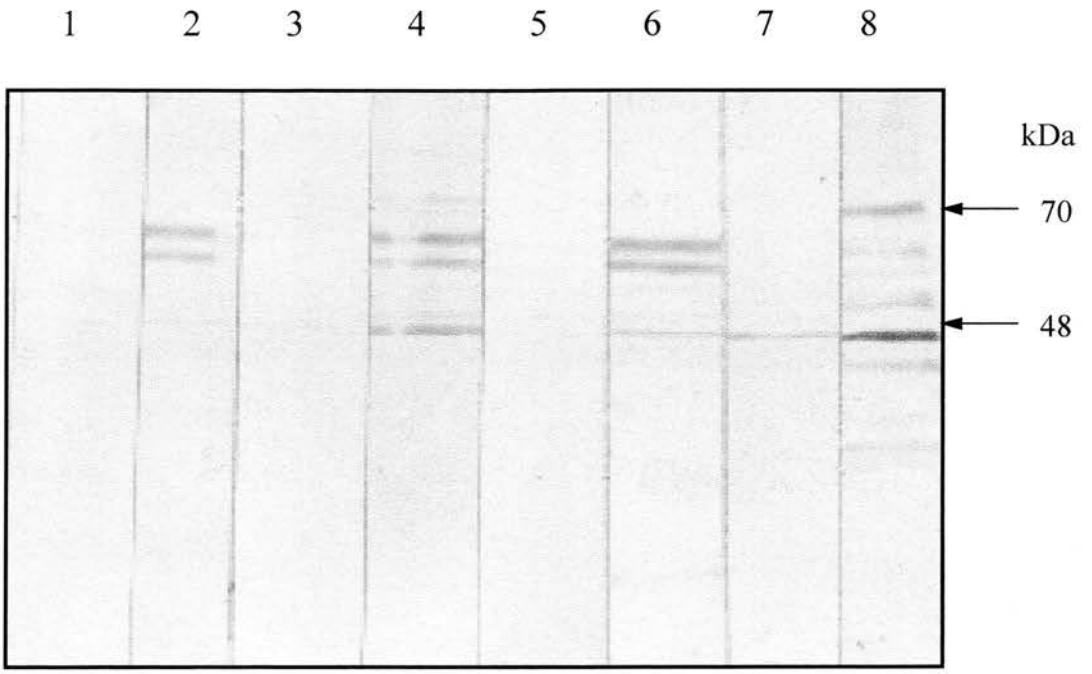


Fig 6.2 Mitochondrial antigens identified in immunoblots by sheep (*E. phagocytophila*) and goat (*C. ruminantium*) antiserum. Lanes 1 and 2, *C. ruminantium* Ball3 strain; Lanes 3 and 4, *C. ruminantium* Gardel strain; Lanes 5 and 6, *C. ruminantium* Welgevonden strain, Lanes 7 and 8, *E. phagocytophila* Feral Goat strain. Lanes 1, 3, 5 and 7 are pre-exposure samples, Lanes 2, 4, 6 and 8 are post-exposure samples containing anti-rickettsial antibodies. Anti-*Cowdria* and anti-*Ehrlichia* antibodies bound to a 48 kDa protein. Two bands consistent with 52-58 kDa were observed in the three strains of *Cowdria* and *E. phagocytophila.* In addition, the sample containing antibodies to *E. phagocytophila* reacted to a band of 70 kDa as observed in Fig 6.1. Arrows indicate the position of the 70 and 48 kDa proteins

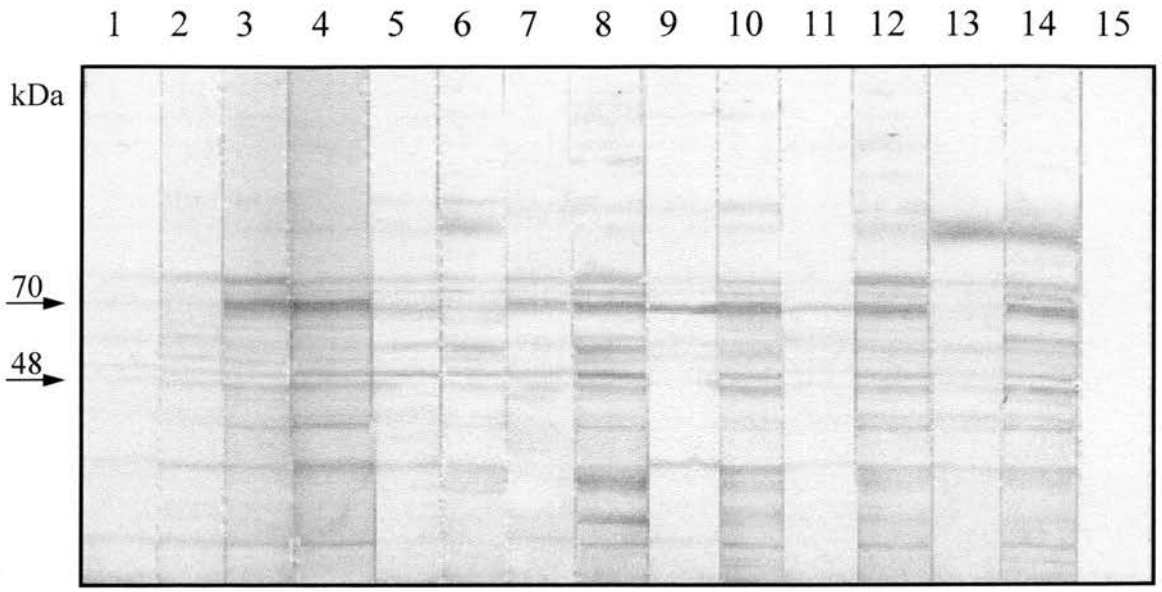


Fig 6.3 Mitochondrial antigens recognised in immunoblots by experimental sheep antiserum before and after challenging the animals with the same strain of *E. phagocytophila*. Even lanes contain post-challenge samples; Odd lanes contain pre-challenge samples; Lane 15 contains pre-inoculation sheep serum. Both pre and post-challenge sera showed anti-mitochondrial activity, stronger reactions were observed in post-challenge sera. Proteins of approx. 48 kDa and 70 kDa (arrows) were recognised by most of the samples as in Fig. 6.1 and 6.2. Many other bands were also observed

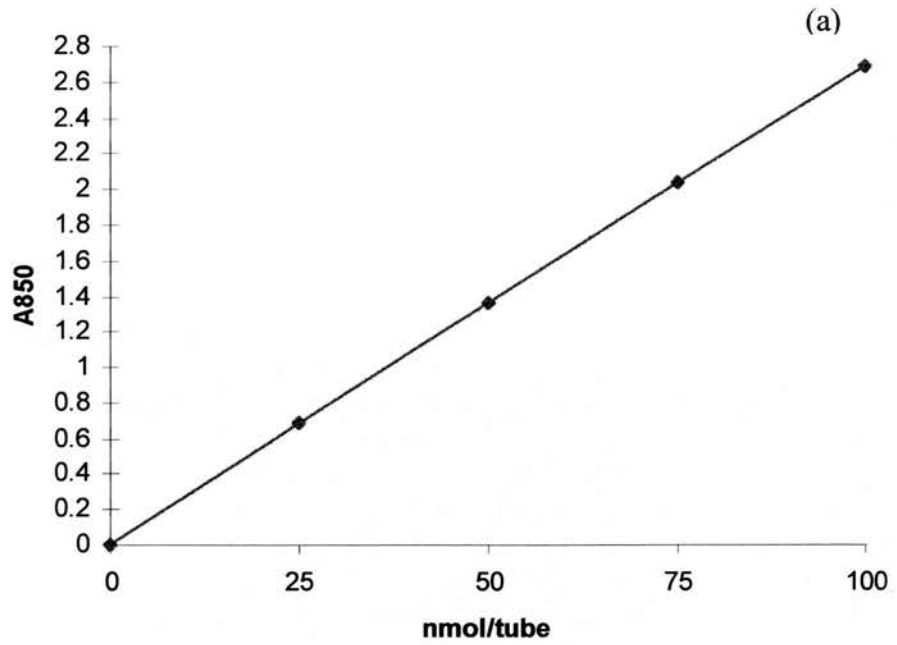
6.3.2 Native gels

Mitochondrial antigenic complexes were identified by BN-PAGE and second dimension tricine SDS-PAGE. Anti-mitochondrial activity varied according to the sample but it seemed to be directed towards proteins in the enzymatic complexes I, II, III, IV and V. Most of the samples bound components of complex V, responsible for the ATPase activity of mitochondria (Dr. Apps, personal communication).

6.3.3 ATPase assays

Fig 6.4 shows the standard curve obtained after measuring normal mitochondrial ATPase activity without any sera.

It was anticipated that the addition to mitochondria of calf serum and pre-exposure samples from experimental sheep would not have any influence in its ATPase activity. The amount of released phosphate from mitochondria alone and mitochondria mixed with serum was compared. Higher amounts of phosphate were observed after the addition of serum, thus the enzymatic activity appeared to be stimulated by its presence. Phosphate release was even higher when the samples contained antibodies to *E. phagocytophila* (Fig 6.5). Contrary to the expected, the presence of anti-rickettsial antibodies appeared to stimulate the phosphate release in vitro from ATP when the absorbance was measured at 850 nm.



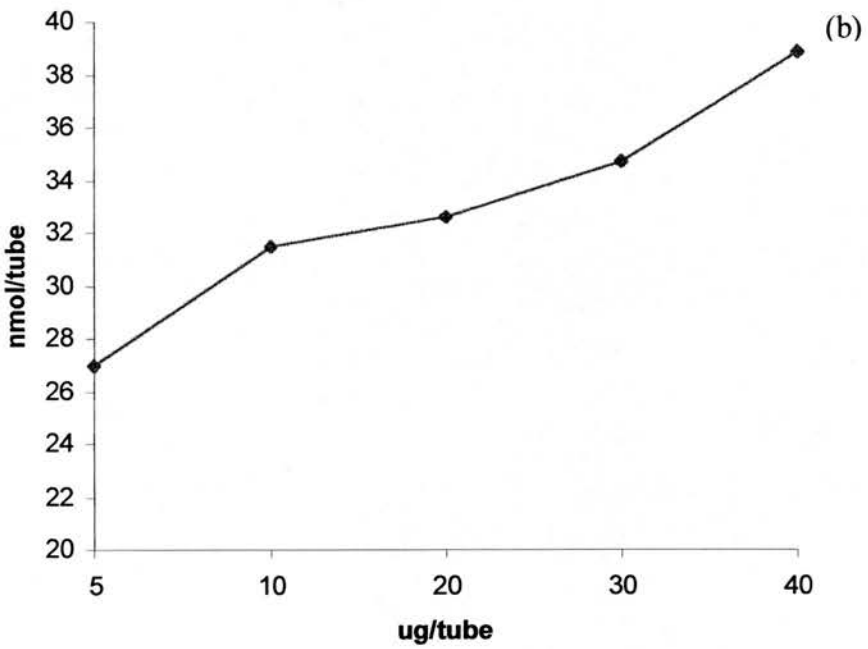


Fig 6.4 (a) The absorbance at 850 nm of known amounts of phosphate was measured and a standard curve was obtained (b) An estimate of the amount of phosphate released from mitochondrial ATP was calculated using the slope and intercept of the standard curve

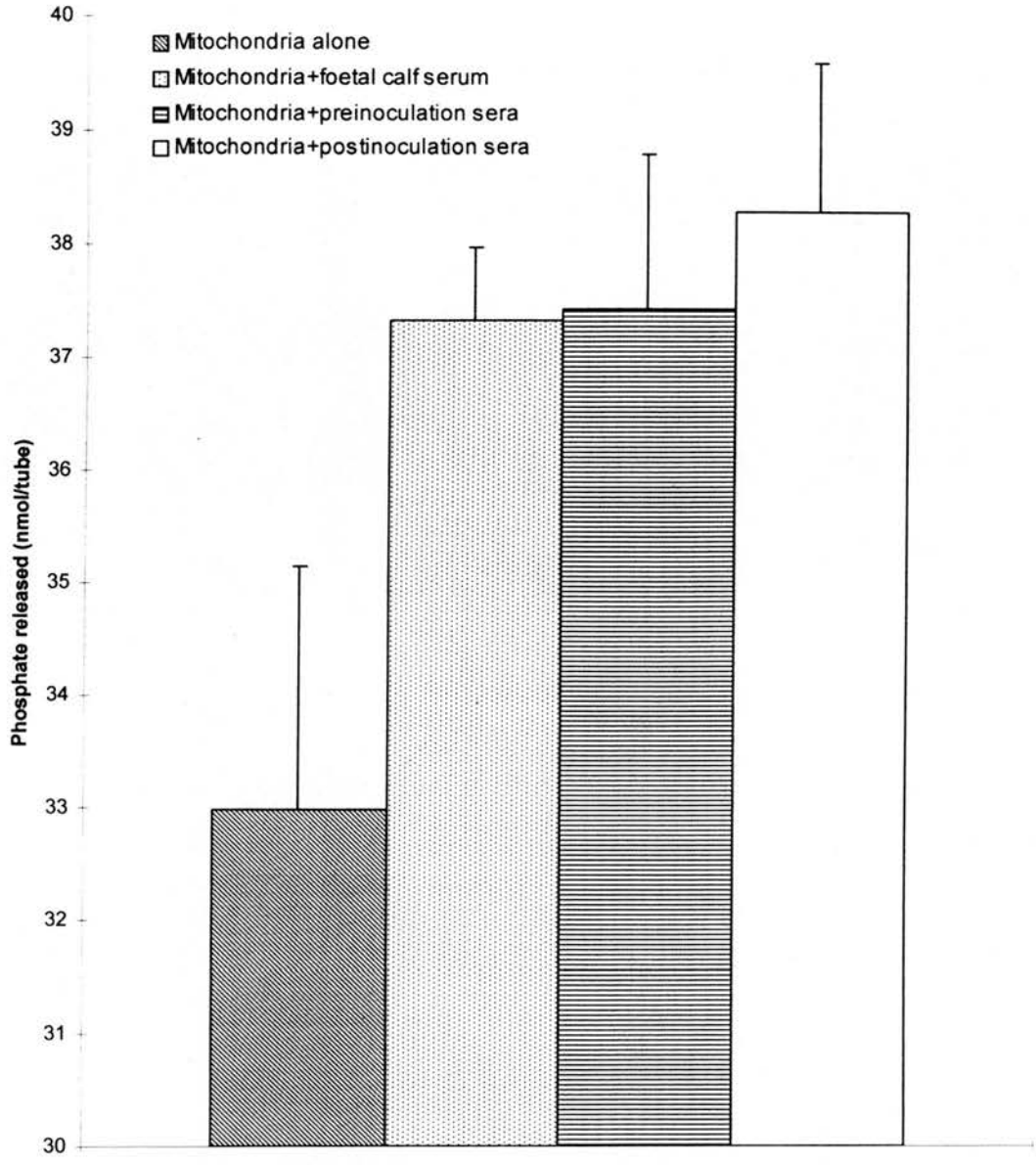


Fig 6.5 Release of phosphate from mitochondrial ATP before and after the addition of non-infected and infected serum samples containing antibodies to *E. phagocytophila*. 20 µg of mitochondria per sample were used. The amount of phosphate (nmol/tube) was estimated from the standard curve. Y error bars represent the standard deviation from the mean

6.4 Discussion

Bacteria in the group *Ehrlichia/Rickettsia* are believed to be the last shared ancestor to mitochondria (Viale and Arakaki, 1994). *Ehrlichia phagocytophila* infections are characterised for inducing immunosuppression although antibody production towards the bacteria can persist for as long as two years (Scott, 1984). The role of those antibodies in the pathology of the disease is unknown but since the organism is mainly intracellular, cellular immune responses are expected to play a more relevant role in immunity. Humoral immune responses towards *Ehrlichia* are known to develop slowly in animals and humans (Bakken *et al.,* 1996). Antigenic mimicry between *Ehrlichia* and mitochondria may mask the pathogenic nature of the bacteria allowing it to successfully invade and destroy leukocytes leading thus to immunosuppression. The obligate intracellular location of the pathogen might permit it to evade host immune responses so they can remain in the host inducing a carrier state. That low level of infection triggers a continuous production of both IgM and IgG antibodies (Scott, 1984) proving that the immunity is not sterile and the pathogen is continuously exposed to the host immune system. In contrast, the newly diagnosed human granulocytic infections are known to produce a short-lived immune response in cases reported from the US (Dumler and Bakken, 1996) although some patients may remain seropositive for at least 14 months after the acute phase (Bakken *et al.,* 1994). The immune response is also shorter in cattle than sheep and it appears to be sterile (Hudson, 1950).

Anti-mitochondrial auto-antibodies to the M2 antigenic components may be triggered by rough forms of bacteria that show more fragile cell walls due to the mutations that leave the intracellular microbial peptides exposed, which can be very antigenic (Butler *et al.,* 1993). Infection by pathogenic organisms phylogenetically closely related to the endosymbiont organelles may also stimulate an autoimmune response to the mitochondria due to the antigenic mimicry between foreign bacteria and self-antigens. However, individual host response appears to be essential in the generation of an autoimmune disease. The role played by those self-antigens in the pathogenesis of rickettsial diseases remains to be elucidated. They could induce liver

or renal damage as a consequence of the circulation of auto-immune antibodies in the blood stream targeting tissues with a high percentage of mitochondrial content like the liver, heart or kidneys. However, *Ehrlichia phagocytophila* infections do not appear to have any specific tissue tropism. Campbell *et al.* (1994) detected histopathological lesions in several organs at necropsy including liver and spleen, the severity of which increased with the duration of the disease. This finding suggests that the presence of circulating immune complexes against ehrlichial and mitochondrial common epitopes may enhance the pathogenesis of the disease. Cells may internalise antibodies bound to inappropriately expressed surface antigens inducing cytotoxic effects when impairing key metabolic pathways. It appears of extreme importance to identify antigenic epitopes shared by *Ehrlichia* and mitochondria in order to develop a suitable vaccine that avoids triggering an autoimmune process and stimulates instead adequate host immune responses. Recombinant expression of immunodominant antigens instead of crude lysates of bacteria seems desirable in order to create a recombinant protein with antigenic properties characteristic solely of *Ehrlichia*. In addition, knowledge of mitochondrial antigens may help to study the interactions between antigens and lymphocytes and their role in the pathogenesis of disease.

Naturally occurring mitochondrial antibodies (NOMA) bind antigens of different molecular weight (98, 65, 61, and 58 kDa) when compared to those found in human Primary Biliary Cirrhosis (PBC) (Butler *et al.,* 1993). A low level of NOMA might be expected in healthy animals as demonstrated by the appearance of bands against mitochondrial proteins when using pre-exposure samples. Very early in development the mammalian immune system develops tolerance to ensure that all pre-T-cells capable of self-antigen recognition are silenced (anergy). However, some antigens may fail to get presented because they are not readily exposed due to their intracellular location such as mitochondria. Samples containing antibodies to rickettsial pathogens after experimental inoculation and challenge bound to proteins of approximate molecular sizes 48, 52, 56 and 70 kDa. The most common mitochondrial antigen, M2, in patients with PBC is a family of four major antigenic proteins consistent with those sizes (Butler *et al.,* 1993). This suggests that anti-

rickettsial antibodies were anti-mitochondrial. It appears feasible that ehrlichial infection may lead to a breakdown of self-tolerance forming the basis of an auto-immune disease in genetically predisposed humans and animals, although this clearly deserves further investigation. In addition, serum containing antibodies to *E. phagocytophila* consistently reacted with a 70 kDa band which appears to cross-react with bacterial membrane fractions (Baum, 1995). Under normal circumstances the immune system is able to discriminate between self-peptides of mitochondrial origin and mutant peptides of mitochondrial or bacterial origin. Phylogenetically closely related bacteria may mimic mitochondrial peptides to the extent that the immune system does not recognise them as foreign or overreacts to them after continuous exposure which could lead to autoimmune responses. Anti-mitochondrial antibodies are frequently detected against epitopic configurations of cardiolipin (Baum, 1995) that can be released extracellularly during increased mitochondrial turnover.

Human granulocytic ehrlichiosis is a multisystem illness with hepatocellular injury, pulmonary involvement, central nervous system manifestations, anemia and thrombocytopenia (Bakken *et al.,* 1994). Although PBC is focused on the liver it is in fact a multi-systemic disorder and is believed to be an autoimmune disease. There are anomalies in B-lymphocytes, high levels of IgG3 subclass, IgG and IgM autoantibodies, and very high titres of AMA, antibodies to organ specific targets like thyroglobulin, nuclear antigens, and circulating immuno-complexes that may contain mitochondrial antigens. There are also T-cell abnormalities, functional deficiency and decreased numbers of CD4+ and CD8+ cells in peripheral blood, inappropriate expression of MHC class II that is concerned with presentation of antigen to helper T-cells and restricted to antigen presenting cells such as dendritic cells, macrophages and B-lymphocytes (Butler *et al.,* 1993). Lymphokines may induce aberrant antigen-presenting capacity that it is believed to play a role in autoimmune diseases. In PBC, 95% of the patients have high anti-M2 antibody titres (both IgG and IgM). The E2 component (dihydrolipoamide acetyltransferase) of the major M2 autoantigen of the pyruvate dehydrogenase complex (PDC) can be associated both to the matrix and to the inner mitochondrial membrane. Oxo-acid dehydrogenase complexes (OADC) are all located in loose association with the inner surface of the inner mitochondrial

membrane. They are evolutionarily very conserved. All M2 autoantigens are constituents of the OADC. All anti-mitochondrial antibodies react against constituents of the complex dehydrogenases (Baum, 1995).

It is believed that M2-AMA may have enzymatic inhibition. AMA cross-reacts against prokaryotic antigens homologous to corresponding mitochondrial enzymes. AMA do not seem to play a role in the pathogenic mechanism of PBC. There might be a genetic predisposition to autoimmune diseases due to maternally inherited point mutations in mitochondrially synthesised peptides of Complex I (Baum, 1995). Mitochondrial mutations may trigger immune responses to normal self-peptides due to molecular mimicry. ATPase assays were performed to determine if anti-*Ehrlichia* antibodies would inhibit ATPase activity. No inhibition was found and the presence of antibodies seemed to stimulate the release of phosphate. The increased amount of phosphate found after the addition of foetal calf serum suggests the presence of phosphate traces in the sera. Monocytic *Ehrlichia* can produce ATP in vitro from the metabolism of glutamine and thus are not totally dependant on their host cells for energy (Weiss *et al.,* 1989). In addition, they also appeared to catabolise ATP when no substrate was present. *Ehrlichia phagocytophila* bacteria are known to be present circulating free in plasma and sera (Foggie, 1951) although they are undetectable by light microscopy. It is possible that the serum used in this study contained viable *E. phagocytophila* bodies because samples were obtained 3-4 weeks after experimental inoculation when *Ehrlichia* can still be detected in Giemsa stained blood smears within neutrophils (Stuen *et al.,* 1998). Therefore the presence of antibodies did not inhibit the release of ATP from mitochondria but the *Ehrlichia* own ATP metabolism probably added to the amount detected. However this was an in vitro assay that might not reflect the situation in vivo.

CHAPTER SEVEN, GENETIC DIVERSITY IN GRANULOCYTIC *EHRLICHIA*: 16S rDNA AND *groEL* GENES SEQUENCE ANALYSIS

7.1 Introduction

Ehrlichia (Cytoecetes) phagocytophila and *E. equi* are known pathogens of domestic ruminants and horses in Europe and the United Stated (MacLeod and Gordon, 1933, Foggie and Allison, 1960, Gribble, 1969; Lewis *et al.*, 1975). Granulocytic organisms genetically closely related to *E. phagocytophila* (Johansson *et al.*, 1995; Clark *et al.*, 1996), and *E. equi* (Chen *et al.*, 1994; Greig *et al.*, 1996) have been recently isolated from humans, dogs, and horses. According to 16S rDNA sequence analysis, *E. phagocytophila* and *E. equi* are 99.9-99.8% identical, respectively, to the agent of human granulocytic ehrlichiosis (Anderson *et al.*, 1991; Chen *et al.*, 1994; Johansson *et al.*, 1995) showing 2 and 3 nucleotide differences in a fragment of 1400-bp. Ribosomal RNAs are considered to be useful molecular chronometers as they occur in all organisms, showing high degree of functional constancy (Woese, 1987). However the variation in this gene sequence is very limited for granulocytic species within the genus *Ehrlichia* and does not allow the design of specific primers or oligonucleotide probes to confirm the identity of the PCR products without sequencing.

Different levels of phylogenetic information can be expected when analysing different genes. Heat shock proteins (HSP) encoded by *groE* operon have been widely studied in bacteria and in several rickettsial organisms including *Ehrlichia chaffeensis* (Sumner *et al.*, 1993), *Cowdria ruminantium* (Lally *et al.*, 1995), and granulocytic *Ehrlichia* (Sumner *et al.*, 1997). These are a family of housekeeping proteins widely distributed among eubacteria, which are essential for the survival of the cell. Although this operon is also conserved between species there is a higher level of nucleotide change than in 16S rDNA sequences (Kolbert *et al.*, 1997; Sumner *et al.*, 1997), which thereby increases possibilities for the analysis of strain variation. Kolbert *et al.*(1997) found that the closely related species *E. chaffeensis* and *C. ruminantium* were 75.6 and 75.2% identical to granulocytic species respectively in a fragment of 1.6 Kb.

It is unknown if dog and horse cases occur with infection by ruminant types or if they are biotypes with particular pathogenicity. Early studies with *E. equi* showed that it could be experimentally transmitted to dogs, cats, and non-human primates (Lewis *et al.*, 1975). Little is known about the ability of ovine strains to induce disease in humans although there is evidence that the human granulocytic agent can naturally and experimentally infect horses and sheep (Madigan *et al.*, 1995; Fish *et al.*, 1997). Human infection with *E. phagocytophila*-like organisms has been observed in Slovenia (Petrovec *et al.*, 1997; Lotric-Furlan *et al.*, 1998) and there is serological evidence of human infection in the UK (Sumption *et al.*, 1995). The role of domestic sheep and deer as natural reservoirs of infection remains to be elucidated. Recent studies have identified roe deer as a likely reservoir for *E. phagocytophila* in the UK (Alberdi *et al.*, in press). Rodents and small mammals seem to be also involved (Telford *et al.*, 1996; Ogden *et al.*, 1998).

The close genetic relationship at 16S rDNA level has raised the awareness towards the zoonotic potential of those *E. phagocytophila*-like organisms (Chen *et al.*, 1994). It is also believed that the newly identified granulocytic isolates could be strain variants of *E. phagocytophila.* Further bacterial genomic analysis is needed to confirm the phylogenetic relationship and to reclassify the granulocytic isolates of human and animal origin.

The aim of this study was to develop rapid methods of differentiation between granulocytic isolates in human and animals based on the analysis of granulocytic *Ehrlichia* species sequences deposited in GenBank database and identification of restriction enzymes to produce a specific RFLP pattern. In addition, partial sequences of *groEL* gene obtained from several samples of *E. phagocytophila* from the UK confirmed RFLP results.

7.2 Materials and methods

7.2.1 Total genomic DNA isolation from granulocytic *Ehrlichia*

Genomic DNA from twelve British *E. phagocytophila* blood stabilates was extracted. These included Old Sourhope (OS), R750, Ehr/8, Feral Goat (FG), Harris, Perth, Pennine, Cairn, Aberfeldy, Penrith, R153, and Lephinmore. Samples were removed from the liquid nitrogen tanks where they were stored at -180 °C, and then equilibrated to room temperature. Samples had mainly sheep or goat origin but Ehr/8 and Aberfeldy were obtained from ticks collected in forests inhabited by deer (Alberdi *et al.*, 1998). In addition, DNA from blood from a granulocytic *Ehrlichia* obtained from a febrile, tick infested horse from Scotland, blood from a cow following abortion outbreaks in England, stabilates containing a granulocytic agent considered representative of HGE from an area of index case in New York, and a stabilate of *E. equi* was extracted (Table 7.1). DNA from the blood stabilates and the three granulocytic isolates was obtained using QIAamp Blood Kit (QIAGEN Ltd) protocol following manufacturers' instructions. It involved lysis of the sample followed by Proteinase K treatment, ethanol precipitation and DNA purification using spin columns. Briefly, 200 µl of each blood sample were aliquoted into 1.5 ml microfuge tubes. 25 µl of Proteinase K (final concentration 1.2 mg/ml) and 200µl of Buffer AL (all buffers were provided with the kit) were added to the sample, then it was mixed immediately by vortexing for 15 sec until the pellet was dissolved. Incubation followed at 70°C for 10 min. 210 µl of isopropanol (96-100%) were added to the sample, and mixed again by vortexing. The mixture was applied carefully to QIAamp spin columns placed in 2 ml collection tubes and centrifuged at 6000 *g* for 1 min. After centrifugation QIAamp spin columns were placed in clean 2 ml collection tubes and the tubes containing the filtrate were discarded. 500 µl of Buffer AW were added to QIAamp spin columns then centrifuged at 6000 *g* for 1 min. Another 500 µl of Buffer AW were added then centrifuged again for 1 min at 6000 *g* and then for 2 min at 15800 *g*. After centrifugation QIAamp spin columns were placed in clean 1.5 ml microfuge tubes. The DNA contained in the columns was eluted with sterile distilled water preheated to 70°C, incubated at 20°C for 1 min then centrifuged for 1 min at 6000 *g* and stored.

Table 7.1 Sample information of *E. phagocytophila* and granulocytic *Ehrlichia* material used in this study

	Name	Species	Location	Host	Reference/Source
Scotland (Southwest)	Feral Goat (FG)	*E. phagocytophila*	Galloway	Goat	Scott, 1981
	Ehr/8	*E. phagocytophila*	Galloway	*I. ricinus* ticks	Alberdi *et al.*, 1998
	R153 (Sourhope)	*E. phagocytophila*	Rigg	Sheep	Scott, 1978
	R750 (Sourhope)	*E. phagocytophila*	Rigg	Sheep	Scott 1978
	Old Sourhope (OS)	*E. phagocytophila*	Rigg	Sheep	Foster and Cameron, 1970
	Cairn	*E. phagocytophila*	Glensaugh	Sheep	Scott, 1981
Scotland (North)	Harris	*E. phagocytophila*	Outer Hebrides	Sheep	Scott, 1982
	M1976	Granulocytic *Ehrlichia*	Caithness	Horse	Sandy Clark, SAC Thurso
	M313	Granulocytic *Ehrlichia*	Caithness	Dog	Sandy Clark, SAC Thurso
	M380	Granulocytic *Ehrlichia*	Caithness	Dog	Sandy Clark, SAC Thurso
	Aberfeldy	*E. phagocytophila*	Perthshire	*I. ricinus* ticks	Scott, 1979
	Perth	*E. phagocytophila*	Amulree/ Perthshire	Sheep	Scott, 1978
	Lephinmore	*E. phagocytophila*	Argyllshire	Sheep	Scott, 1978
England	Pennine	*E. phagocytophila*	Cumbria	Sheep	Scott, 1987
	Penrith	*E. phagocytophila*	Cumbria	Sheep	Scott, 1978
	C8	*E. phagocytophila*	Penrith/ Cumbria	Cattle	Philip Watson, VIC Penrith
USA	*Ehrlichia equi*	*E. equi*	Louisiana	Horse	R. Corstvet, Louisiana State University
	HGE	Agent of HGE	New York	Human	Telford *et al.*, 1996
Netherlands	Ameland	Granulocytic *Ehrlichia*	Ameland Islands	Goat	Jongejan *et al.*, 1989
	NR2	Granulocytic *Ehrlichia*	Ameland Islands	Cattle	Frans Jongejan, Utrecht
	Canine Utrecht	Granulocytic *Ehrlichia*	-	Dog	Frans Jongejan, Utrecht

7.2.2 Nested PCR to obtain a 813-bp fragment of the *groE* gene for 18 different *E. phagocytophila*-like isolates

Heat shock protein (HSP) specific primers, HSP354 and HSP2165 followed by primers HSP961 and HSP1754a, were used to amplify an 813-bp fragment of the *groEL* operon gene based on the HGE published sequence (Kolbert *et al.*, 1997) for the 12 *E. phagocytophila* isolates, the HGE agent, *E. equi*, a *E. phagocytophila*-like organism isolated from a horse from Scotland, *E. phagocytophila* from TBF cases in cattle from England, and two Dutch granulocytic *Ehrlichia* isolated from goats and cattle (Table 7.1). 0.2 µM of each primer was incorporated into a PCR master mixture containing 0.5 mM Mg Cl_2 (Sigma, Poole, UK), 0.2 mM of each dNTP (Pharmacia Biotech, USA), 1.25 U of Ultrotaq DNA polymerase, and 10x Ultrotaq buffer (ThermoMetric Ltd., Northwich, UK). Amplification was performed in a 50 µl reaction volume, overlaid by 50 µl of mineral oil, within a Hybaid OmniGene thermal cycler (Hybaid Ltd, Middlesex, UK) using the conditions previously described (Kolbert *et al.*, 1997). The first amplification reaction consisted of three cycles of denaturation at 94°C for 1 min, annealing at 58°C for 2 min, and extension at 70°C for 1 min 30 sec, followed by 37 cycles of denaturation at 90°C for 1 min, annealing at 68°C for 1 min, and extension at 70°C for 1 min 30 sec, followed by a final extension at 68° C for 7 min. The second amplification reaction was as follows. An initial denaturation step at 94°C for 4 min was followed by 40 cycles of denaturation at 94°C for 45 sec, annealing at 62°C for 45 sec, and extension at 72°C for 45 sec, followed by a final extension at 72° C for 7 min to ensure that Taq polymerase added a single deoxyadenosine (A-overhang) to the 3'-ends of all duplex molecules after PCR. Subsequent to amplification, 10 µl of the PCR products were electrophoresed in a 1% agarose gel stained with ethidium bromide (0.3 ng/µl).

The presence of a granulocytic *Ehrlichia* in the blood of the horse from Scotland was confirmed by primers and amplification conditions considered to be specific for *E. phagocytophila*-like organisms (Munderloh *et al.*, 1996b) at 16S rRNA gene level to obtain an 151-bp product as described in Chapter Three.

7.2.3 Cloning of an 813-bp amplicon from Feral Goat, Ehr/8, Perth and Harris blood stabilates, Caithness horse, Penrith cattle isolates, and two canine samples from Scotland (M380, M313)

Amplicons obtained by nested PCR of six samples (Harris, Perth, Ehr/8, Feral Goat, and the bovine and equine granulocytic *Ehrlichia* isolates) were cloned using TA Cloning® Kit (Invitrogen BV, The Netherlands), following manufacturers' protocols. This procedure does not require any enzymatic modification of the PCR product or PCR primers containing restriction sites. Briefly, the PCR product from the reaction mixture was ligated into pCR® 2.1 vector (Invitrogen) which contains single 3' T-overhangs that allows direct ligation into the 3' A-overhang of the PCR product. Each ligation reaction contained 10x ligation buffer, 25 ng/µl of the vector, 10ng of fresh PCR product, T4 DNA ligase, and sterile distilled water to a final volume of 10 µl. After overnight incubation at 14°C, 2 µl of the ligation reaction were transformed into One Shot™ *Escherichia coli* competent cells containing 0.5M β-mercaptoethanol. The mixture was incubated on ice for 30 min then heat shocked at 42°C for 30 sec. 450µl of SOC medium (Appendix C) were added and the solutions were placed shaking at 225 rpm, 37°C, for an hour. 50µl and 200µl of the transformation products were plated out in Luria Bertani agar plates (Appendix C) containing 50 mg/ml of Ampicillin and X-Gal (Promega). X-Gal allowed the differentiation of the colonies that contained the insert (white) from the colonies that did not contain it (blue). The plates were incubated at 37°C for at least 18h and then shifted to 4°C for colour development of the colonies. Single white colonies were selected and placed in tubes containing 3 ml of Luria Broth Medium (Appendix C) and 50 µl of Ampicillin (0.8 µg/µl) then grown overnight in an incubator shaking at 37°C.

Two canine samples (M380, M313) were submitted (VIC, Thurso) because their clinical signs were consistent with granulocytic *Ehrlichia* infection, including the observation of intraneutrophilic inclusions in Giemsa stained blood smears. After nested PCR amplification the products were also cloned and sequenced as for the rest of the samples.

7.2.4 DNA purification using Wizard minipreps

Plasmid DNA was isolated from pCR® 2.1 vector using Wizard® Plus Minipreps DNA Purification System (Promega) according to manufacturers' instructions. Briefly, 1.5 ml of single colonies grown overnight were aliquoted and centrifuged at 15800 *g* for 15 min. The supernatant was poured off and the tube was blotted upside-down on a paper towel to remove excess media. The cell pellet was resuspended in 200µl of Cell Resuspension Solution (50 mM Tris, 10 mM EDTA, 100 µg/ml Rnase A). 200µl of Cell Lysis Solution (0.2 M NaOH, 1% SDS) were added and mixed with the cell suspension then 200µl of Neutralisation Solution (1.32 M potassium acetate) were incorporated and the sample mixed by gently inverting the Eppendorf tubes. The lysate was centrifuged at 11000 *g* for 5 min then the supernatant transferred into a barrel of the Minicolumn/Syringe assembly containing 1ml of Purification Resin and vacuum was applied to pull the resin/lysate mix into the Wizard™ Miniprep Column. 2 ml of Column Wash Solution (80 mM potassium acetate, 8.3 mM Tris-HCl, 40 µM EDTA, 55% ethanol) were added to the Syringe Barrel and the vacuum was reapplied until the solution was drawn through the Minicolumn. The Syringe Barrel was removed and the Minicolumn transferred to a 1.5 ml Eppendorf tube. The Minicolumns were centrifuged at 15800 *g* for 30 sec to remove any residue of Column Wash Solution and placed in clean 1.5 ml Eppendorf tubes. Purified DNA was eluted with 50 µl of water, incubated for at least 1 min, then centrifuged for 30 sec at 15800 *g*. The samples were kept on ice until use. The presence of the purified insert was confirmed by restriction digestion. The samples were incubated at 37°C for at least one hour with 12 units of EcoRI enzyme (1 µl enzyme, 1 µl buffer, 8 µl sample), then electrophoresed in 1% agarose gels.

Following cloning, amplification products from the six isolates were sequenced in both directions using automated sequence analysis (I. Bennett, Royal Dick School of Veterinary Studies, University of Edinburgh). The respective sequences were aligned with each other in order to resolve sequence ambiguities and a corrected sequence was obtained.

Blood stabilate DNA extracts from the following isolates R153, R750, Feral Goat, Ehr/8, Harris, Perth, Penrith, Pennine, Aberfeldy, and Cairn were sent to Utrecht University for 16S rDNA sequence analysis.

7.2.5 Computer analysis of sequence data of *groEL* amplicons from granulocytic *Ehrlichia* isolates from Britain

The corrected sequences were edited and comparisons were made using GCG (Genetics Computer Group, 1994) and Lasergene DNAstar software packages.

7.2.6 PCR and cloning of a canine sample from Utrecht using Lasergene designed primers to amplify a 410-bp DNA fragment of *groE* gene.

GroEL operon gene sequences for *E. phagocytophila* and closely related organisms were retrieved from Genbank™ computer database and compared. Specific oligonucleotide primers for *E. phagocytophila* were designed using Lasergene DNAstar software program to amplify a 410-bp PCR product. Primers were synthesised by Cruachem (Glasgow) with the following sequences

HSP534 5' TGTACTCAATAAGCTCCGTGGTG 3' and
HSP1326 5' CTACTCTGTCTTTGCGTTCCTTCA 3' (Appendix 3.1)

DNA was extracted using a QIAamp Blood Kit (QIAGEN Ltd), as previously described, from *E. phagocytophila* experimentally infected sheep blood when its parasitaemia reached 32% as determined by examination under oil immersion of blood smears stained by the Giemsa method, to use it as a positive control. The detection threshold of the primers was determined by 10-fold serial dilutions of DNA in sterile distilled water to detect the minimum number of infected neutrophils needed to obtain a positive signal.

A canine sample from Utrecht (Netherlands) which showed rickettsial bodies in granulocytic cells in a blood smear slide, was kindly provided by Dr. Cornelis Bekker. It was amplified using the above described primers under the following conditions. Three cycles of denaturation at 94°C for 1 min, annealing at 58°C for 2 min, and extension at 70°C for 1 min 30 sec, were followed by 37 cycles of

denaturation at 90°C for 1 min, annealing at 68°C for 1 min, and extension at 70°C for 1 min 30 sec, followed by a final extension at 68° C for 7 min.

After PCR amplification the canine sample was cloned and sequenced as described in section 7.2.3.

7.2.7 Isolate characterisation by digestion with restriction endonucleases

MboII (New England Biolabs, Hitchin, UK) and HaeIII (Sigma, Poole, UK) endonucleases were used to confirm the cleavage sites predicted from the *groEL* sequences deposited in Genbank™ database (Sumner *et al.*, 1997), and the sequences obtained in this study. Ten µl of the PCR product after second round of amplification with HSP961 and HSP1754a primers were mixed with 2.5 U of MboII enzyme (5 U of HaeIII), 7.5 µl of MilliQ water, and 2 µl of the recommended buffer. Samples were incubated at 37 °C for an hour, then 10 µl of the reaction were assessed by polyacrylamide gel electrophoresis (Appendix C) followed by silver staining based on the method of Sambrook *et al.* (1989). Briefly, polyacrylamide gels were cast as previously described for SDS-PAGE of discontinuous gels using the BioRad system, then run at 100 V for 30 min until the dye buffer reached the end of the gel. Gels were recovered and fixed for 10 min in 100 ml of fixative buffer containing 10% ethanol/ 0.5 % glacial acetic acid. After a brief wash in distilled water, gels were stained for 10 min with 11mM silver nitrate, then the colour developed with 0.75 M NaOH solution containing 0.75 ml formaldehyde per 100 ml. The reaction was stopped with 0.07 M solution of Na_2CO_3 when the desired level of colour was achieved.

7.2.8 GenBank accession numbers

The accession numbers for the *groEL* nucleotide sequences used for comparisons are as follows: U96730 for *E. phagocytophila* Old Sourhope; U96729 for *E. phagocytophila* Feral Goat; U96735 for *E. phagocytophila* from a Swiss horse; U72628 and U96728 for the agent of HGE in the United States; AF033101 for the agent of HGE in Europe; U96727 for *E. equi*.

7.3.1 Nested PCR, cloning and DNA purification

An 813-bp fragment of *groEL* gene was successfully amplified after nested PCR from all the samples (Fig 7.1) but for two that were labelled 'Deer' and 'Rigg', no products were obtained even after PCR was repeated several times for the last two samples. Amplicons from six of the isolates (Feral Goat, Ehr/8, Perth, Harris, the granulocytic *Ehrlichia* isolate from a horse from Scotland, and cattle from England) were subsequently cloned and sequenced (Fig 7.2). Blood samples were received from two dogs from the North of Scotland (M380, M313) which showed neutrophil inclusions in blood smears. After PCR amplification their products appeared to have half the expected size (approximately 400-bp, Fig 7.3) of the controls but they were also included for cloning and sequencing.

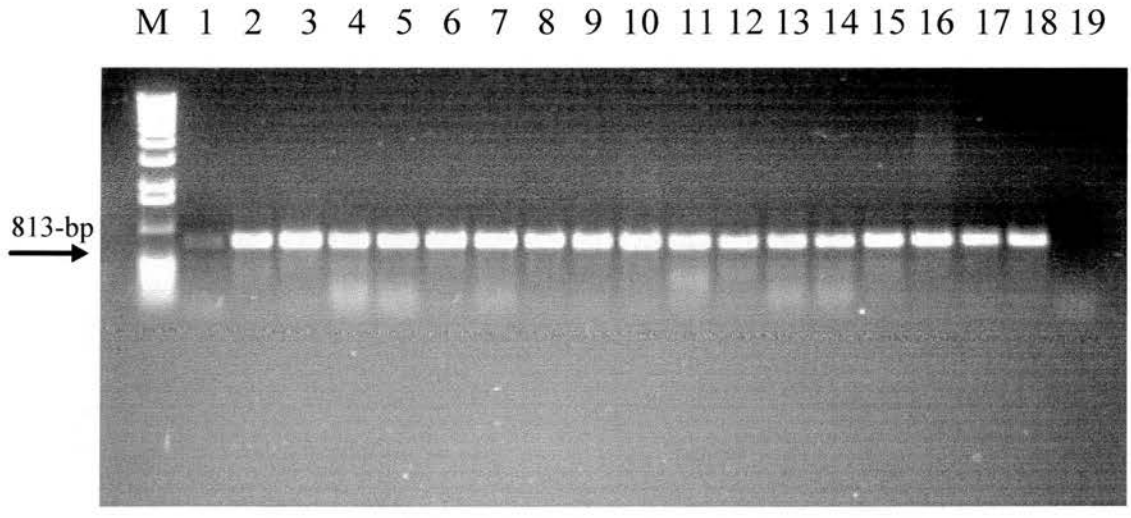


Fig 7.1 Granulocytic *Ehrlichia* nested PCR of *groEL* operon using specific primers. Arrow indicates position of the 813-bp PCR products. M, 1 Kb molecular size marker. Lane 1, HGE; Lane 2, *E. equi*; Lane 3, C8; Lane 4; Caithness horse; Lane 5, Aberfeldy, Lane 6, Cairn; Lane 7, Ehr/8; Lane 8, Feral Goat; Lane 9, Harris; Lane 10, Lephinmore; Lane 11, NR1; Lane 12, NR2; Lane 13, Old Sourhope; Lane 14, Pennine; Lane 15, Penrith; Lane 16, Perth; Lane 17, R153; Lane 18, R750; Lane 19, sterile distilled water.

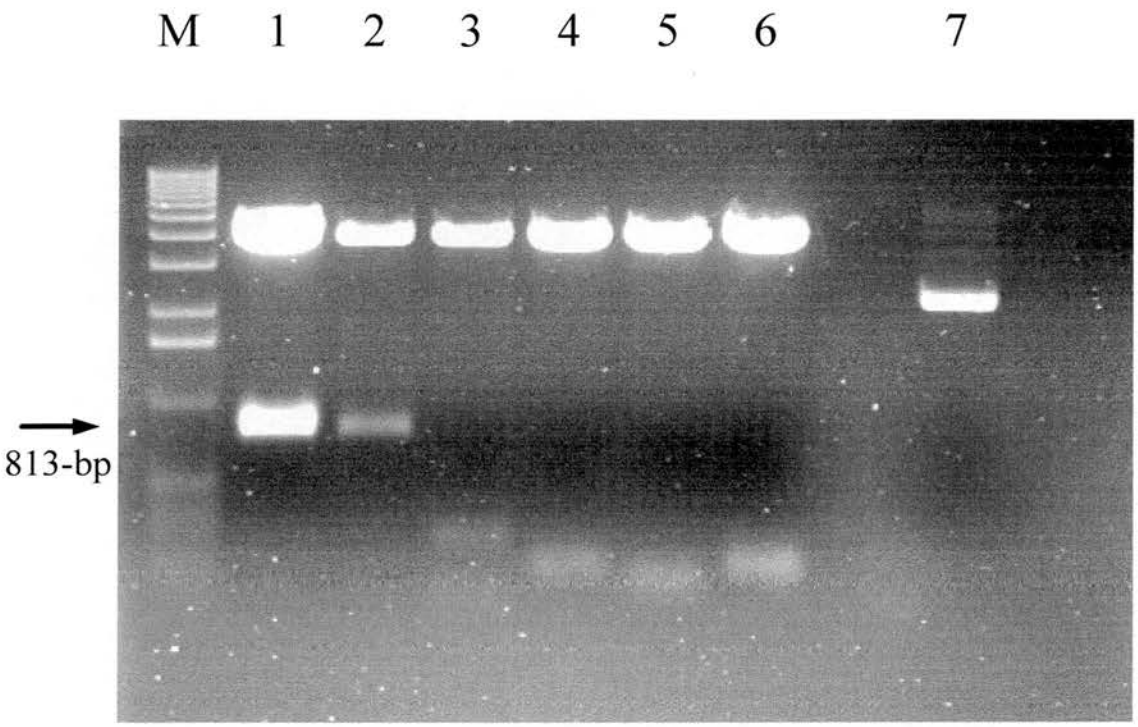


Fig 7.2 Ethidium bromide stained agarose gel (1%) showing the 813-bp insert (Perth isolate) after purification and digestion from the plasmid with EcoRI, ready for sequencing. Lane M, 1Kb molecular size marker; Lanes 1-2, plasmids containing the insert; Lanes 3-6, plasmids that did not contain the insert; Lane 7, undigested plasmid (supercoiled).

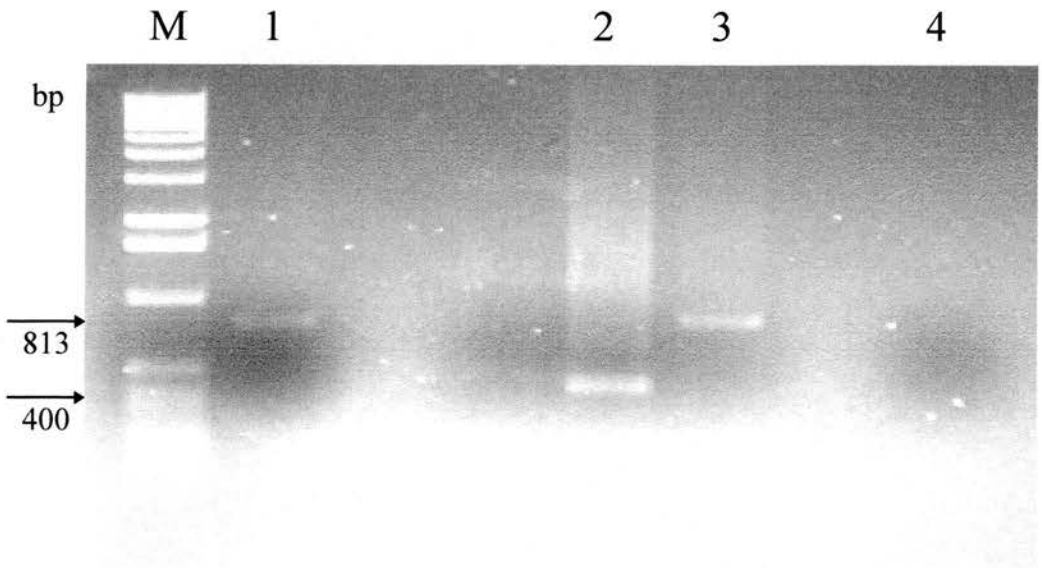


Fig 7.3 Granulocytic *Ehrlichia* nested PCR of *groEL* operon using specific primers. Arrows indicate position of 813 and 400-bp (approx.) PCR products. M, 1 Kb molecular size marker. Lane 1, Feral Goat; Lane 2, canine sample M313; Lane 3, bovine sample C8; Lane 4, sterile distilled water

7.3.2 Sequence analysis and alignment of *groEL* amplicons from granulocytic *Ehrlichia* isolates from Britain

On average 5 nucleotide differences were observed between the published sequences of HGE agent and the 6 different isolates of granulocytic *Ehrlichia* from Britain (Table 7.2). The nucleotide differences did not represent any aminoacid variation in the isolates of North American origin. Nucleotide substitutions did however alter the deduced amino acid sequence in some of the European isolates (Table 7.3). A dendrogram showing the alignment of *E. phagocytophila*-like samples is shown in Fig 7.4 and the percent similarity between samples is shown in Table 7.4. Sequence analysis of the two canine samples from Scotland revealed no similarity to *E. phagocytophila* or any granulocytic *Ehrlichia* and they were considered non-specific PCR amplifications.

Table 7.2 Nucleotide differences observed between granulocytic *Ehrlichia* spp. of human, ruminant and equine origin in a fragment of 813-bp (including primers) from the *groEL* gene. Nucleotide position was based on the partial *groESL* sequence of *E. equi* (GenBank accession No. U96727). Relevant changes in the sequence are underlined. Changes in position 848 allowed the differentiation between European samples when using MboII and HaeIII. Changes in position 965 produced the different cutting patterns between European and North American samples by using MboII

	Nucleotide position											
Isolate	560	566	603	729	776	848	890	891	956	965	1005	1049
E. equi	G	G	T	A	C	A	G	A	C	A	G	C
HGE[a]	-	-	-	-	-	-	-	-	-	-	-	-
HGE[b]	-	-	-	-	-	-	-	-	-	-	-	-
HGE[c]	-	-	-	-	T	-	-	-	-	G	-	-
Swiss horse	-	-	-	-	T	-	-	-	-	G	-	-
Scottish horse	-	-	-	-	T	-	-	-	-	G	-	-
Canine Utrecht	na	na	na	na	na	na	na	na	-	G	-	-
Ehr/8	-	A	-	-	-	-	-	-	-	G	-	-
Penrith cattle	-	A	-	G	-	-	-	-	T	G	A	T
Feral Goat 1	-	A	-	-	-	-	-	-	T	G	-	T
Feral Goat 2	-	A	C	-	-	G	-	-	-	G	-	-
Old Sourhope	-	A	-	-	-	G	T	-	T	G	-	-
Harris	A	A	-	-	-	G	T	-	T	G	-	-
Perth	-	A	-	-	-	G	T	G	T	G	-	T

Table 7.2 Cont.

Isolate	Nucleotide position											
	1085	1088	1101	1112	1118	1193	1229	1238	1244	1289	1308	1316
E. equi	G	A	G	T	T	T	G	C	C	G	G	A
HGE[a]	-	-	-	-	-	-	-	-	-	-	-	T
HGE[b]	-	-	-	-	-	-	-	-	-	-	-	-
HGE[c]	A	C	-	C	-	-	A	-	T	-	-	G
Swiss horse	A	C	-	C	-	-	A	-	T	-	-	G
Scottish horse	A	C	-	C	-	C	A	-	T	-	-	G
Canine Utrecht	A	C	A	C	-	-	A	-	T	C	T	na
Ehr/8	A	C	-	C	C	-	A	-	T	-	-	G
Penrith cattle	A	C	-	C	-	-	-	-	T	-	-	G
Feral Goat 1	A	C	-	C	-	-	-	-	-	-	-	G
Feral Goat 2	A	C	-	C	C	-	-	-	-	-	-	G
Old Sourhope	A	C	-	C	C	-	A	-	T	-	-	G
Harris	A	C	-	C	C	-	A	-	T	-	-	G
Perth	A	C	-	C	-	-	-	T	-	-	-	G

[a] Kolbert *et al.*, 1997; GenBank accession No. U72628

[b] Sumner *et al.*, 1997; GenBank accession No. U96728

[c] Lotric-Furlan *et al.*, 1998; GenBank accession No. AF033101

\- Indicates same base pair as *E. equi*

na not available

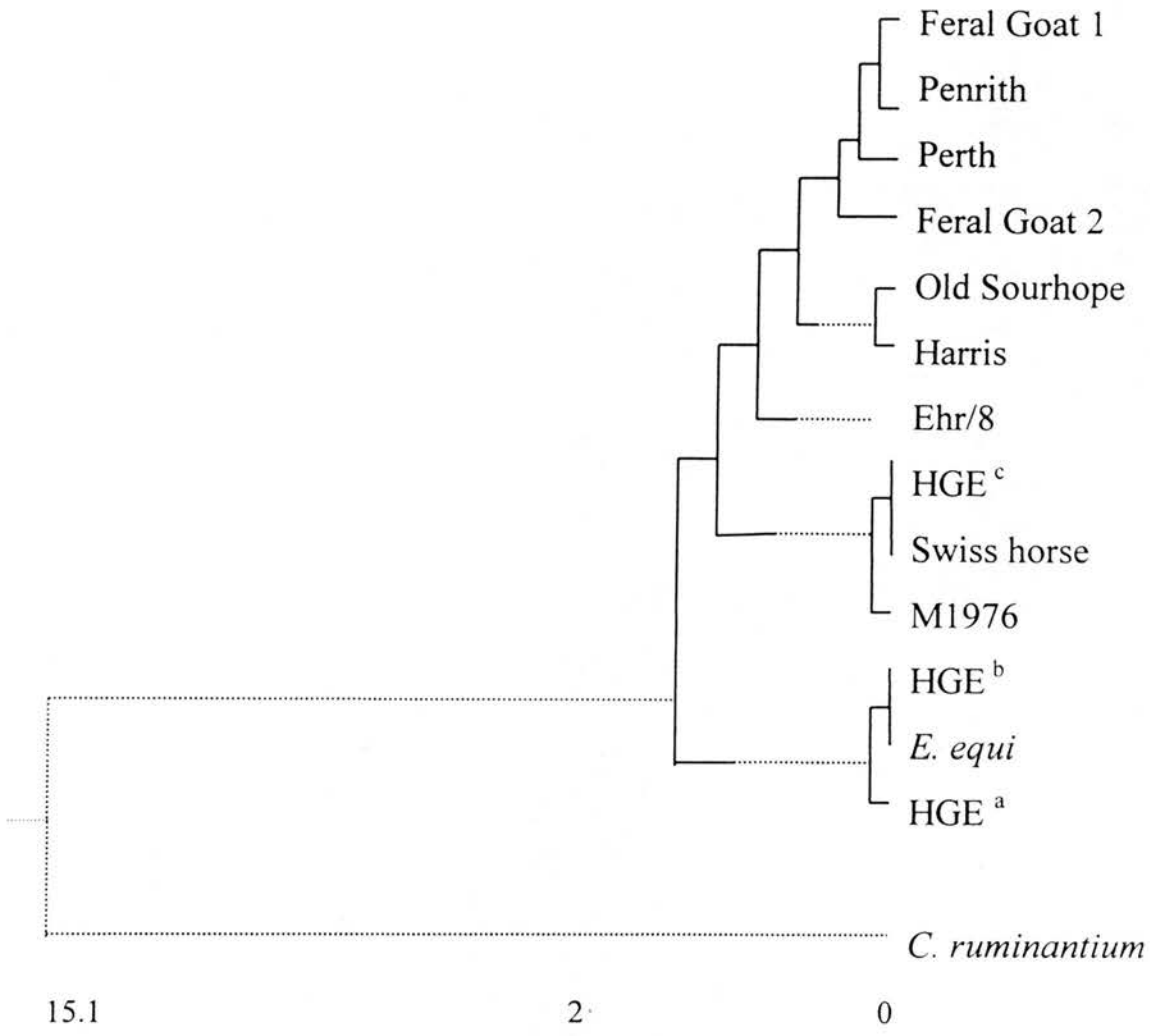
Table 7.3 Altered amino acid sequence in European isolates by nucleotide substitutions in an 813-bp fragment of the *groEL* gene. Amino acid position relative to *E. equi* sequence (GenBank accession No. U96727)

	Amino acid position				
	140	172	205	245	259
E. equi	D	C	T	P	S
HGE	-	-	-	-	-
Swiss horse	-	-	-	-	-
Scottish horse	E	-	-	-	-
Ehr/8	E	-	-	T	-
Penrith cattle	E	-	A	-	-
Feral Goat 1	-	-	-	-	-
Feral Goat 2	E	R	-	-	-
Old Sourhope	-	-	-	-	-
Harris	E	-	-	-	G
Perth	E	-	-	-	-

- Indicates same amino acid as *E. equi*

Table 7.4 Percent similarity of *groEL* partial sequences (813-bp) using Clustal method. A human granulocytic isolate from Europe appears identical to a horse-derived sample from Switzerland but they both differ from *E. equi* and North American samples of HGE

% Similarity														
Species	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1. Ehr/8	100	99.4	99.5	99.6	99.5	99.1	98.8	98.8	99.8	99.6	99.5	99.6	98.8	71.5
2. Feral Goat 1		100	99.4	99.2	99.1	99.7	99.5	99.0	99.0	99.2	99.1	99.2	99.0	71.7
3. Feral Goat 2			100	99.4	99.2	99.1	99.1	98.8	98.8	99.1	99.0	99.1	98.8	71.0
4. Old Sourhope				100	99.9	99.0	99.2	98.4	98.4	99.2	99.1	99.2	98.4	71.7
5. Harris					100	98.8	99.1	98.3	98.3	99.1	99.0	99.1	98.3	71.7
6. C8						100	99.2	98.7	98.7	99.0	98.8	99.0	98.7	71.3
7. Perth							100	98.4	98.4	98.7	98.6	98.7	98.4	71.5
8. HGE[a]								100	99.9	99.0	98.8	99.0	99.9	71.7
9. HGE[b]									100	99.0	98.8	99.0	100	71.5
10. HGE[c]										100	99.9	100	99.0	71.9
11. M1976											100	99.9	98.8	71.8
12. Swiss horse												100	99.0	71.8
13. *E. equi*													100	71.5
14. *C. ruminantium*														100



[a] Kolbert *et al.*, 1997; GenBank accession No. U72628
[b] Sumner *et al.*, 1997; GenBank accession No. U96728
[c] Lotric-Furlan *et al.*, 1998; GenBank accession No. AF033101

Fig 7.4 Dendrogram generated with Lasergene DNAstar programme using Clustal method from an alignment of a fragment of 813-bp of *groEL* operon illustrating the phylogenetic relationship between several granulocytic *Ehrlichia* isolates. *Cowdria ruminantium groE* partial sequence was included for comparison. Numbers at the bottom of the tree indicate number of substitution events

7.3.3 16S rDNA sequencing results

16S rDNA sequence analysis from the following samples R153, R750, Feral Goat, Ehr/8, Harris, Perth, Penrith, Pennine, Aberfeldy, and Cairn revealed that they were almost identical to the HGE agent and *E. equi*. Differences were found however at relevant positions (Table 7.5). Harris and R750 isolates appeared to be identical to the HGE but for one nucleotide change at position 91.

Table 7.5 Nucleotide differences between North American and European isolates found in 16S rRNA gene sequences

	Nucleotide position			
	49	78	91	98
E. equi	C	A	A	A
HGE	T	-	-	G
R153	T	G	-	-
R750	T	-	G	G
FG	T	-	-	-
Ehr/8	T	-	-	-
Harris	T	-	G	G
Perth	T	-	-	-
Penrith	T	-	-	-
Pennine	T	-	-	-
Aberfeldy	T	-	-	-
Cairn	T	-	-	-

- Indicates same nucleotide as *E. equi*

7.3.4 Development of rapid methods of differentiation between granulocytic *Ehrlichia*

The nucleotide variation between the published sequences of HGE, *E. equi*, and *E. phagocytophila* allowed to determine various restriction enzyme cleavage sites that could enable differentiation between agents in the *E. phagocytophila* genogroup. Amplicons from 18 different *E. phagocytophila*-like organisms were processed by enzymatic restriction and their banding patterns compared to the *E. phagocytophila* isolates of known sequence. Amplicons from the agents of HGE and *E. equi*, both of North American origin, could be readily distinguished by MboII cleavage from the European granulocytic agents (Fig 7.5 and 7.6). The former samples showed the expected bands, which migrated consistent with sizes of 264,

146, 121, 97, 80, and 56 bp. European samples had an additional band of 177 bp because of the absence of the restriction site between the 97 and 80 bp bands, which were consequently absent (Fig 7.6.c). In addition, some of the European isolates (Ameland, Perth, Aberfeldy, Harris, Old Sourhope, and R750) gave a distinct pattern from the rest showing an additional 79 bp band. The remaining fragments (up to 813-bp) were too small to be resolved on the gel. MboII also cut in position 546 (included in the primer) in Penrith cattle, Ehr/8, Harris, Perth and Scottish horse samples and position 856 in all the European samples except Harris, Perth and Old Sourhope. MboII did not cut in position 976 in samples of European origin allowing the differentiation from the North American ones. Fig 7.6 shows the location of primers and MboII restriction sites for the 813-bp *groEL* gene fragment.

RFLP differences were also detected between the same set of isolates with HaeIII restriction enzyme (data not shown). The same European samples that had an additional 79-bp band (after MboII restriction digestion) showed three bands of 423, 317, and 73 bp. When compared, the rest of the samples, including the ones of North American origin, showed only two bands consistent with the predicted fragment size of 423 and 390 bp.

A 410-bp fragment derived from a dog from Utrecht (section 7.3.5) was also digested using MboII enzyme. It showed a pattern as expected from an *E. phagocytophila*-like organism.

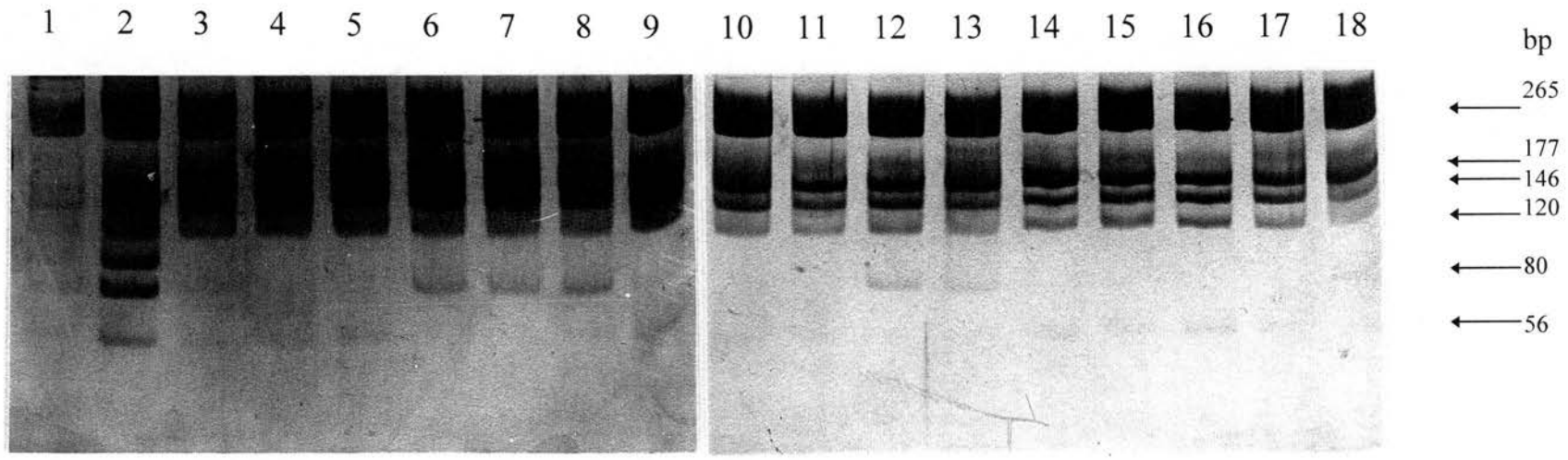


Fig 7.5 Acrylamide gel stained with silver nitrate showing the results after MboII endonuclease restriction digestion. Size of the fragments is indicated in base pairs (arrows). Lanes: 1, HGE; 2, *E. equi*; 3, Feral Goat; 4, Lephinmore; 5, horse from Scotland; 6, Ameland; 7, Perth; 8, Aberfeldy; 9, Cairn; 10, C8; 11, NR2; 12, Harris; 13, Old Sourhope; 14, Pennine; 15, Penrith; 16, Ehr/8; 17, R153; 18, R750.

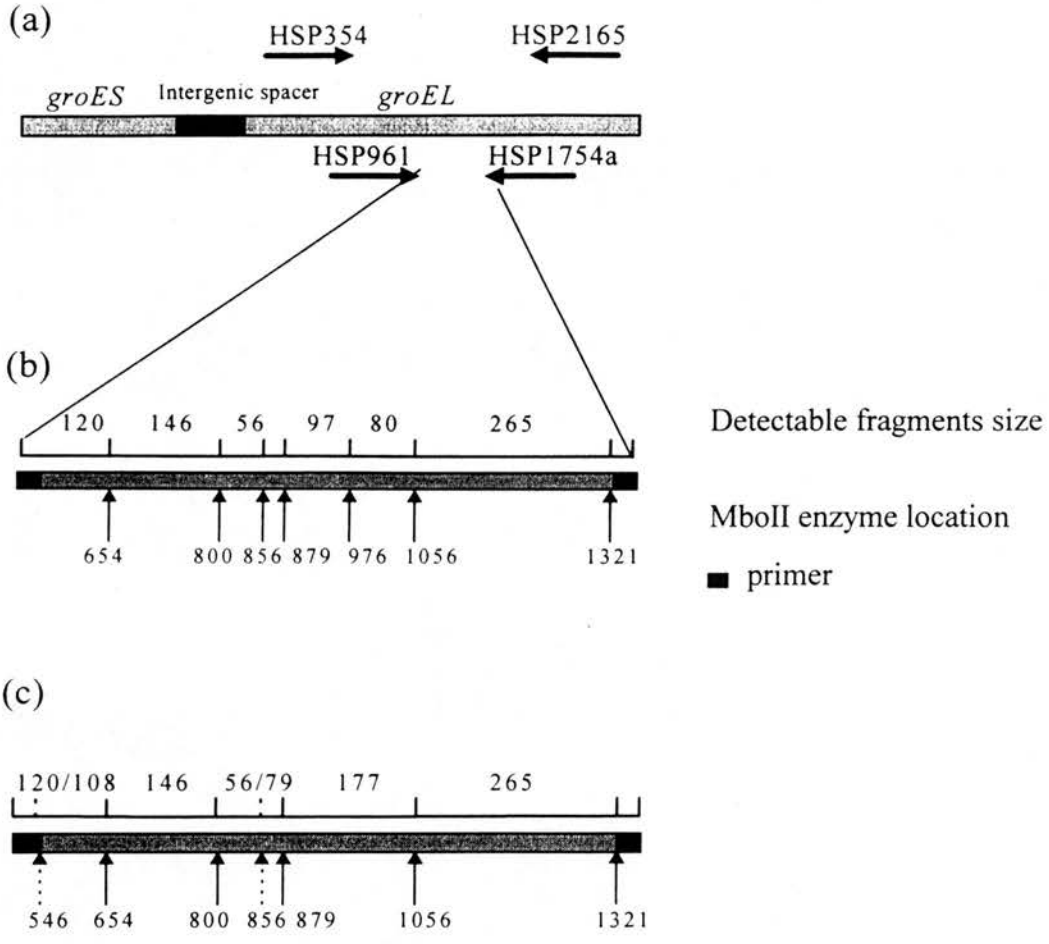


Fig 7.6 (a) Location and orientation of PCR primers with respect to *groESL* heat shock operon. Location of MboII restriction sites and size of fragments detectable in the acrylamide gel for *groEL* partial sequences from (b) North American and (c) European samples. Sequence starts at position 534 and ends at position 1326 relative to *E. equi* sequence deposited in GenBank database (Accession number U96727)

7.3.5 PCR and cloning of a canine sample from Utrecht using Lasergene designed primers to amplify a 410-bp DNA fragment of *groE* gene.

PCR specific primers amplified a 410-bp product from *E. phagocytophila* DNA but they did not amplify DNA from the closely related species *Cowdria ruminantium*, *E. bovis* and *E. canis* when they were used as templates. Interestingly, they also amplified DNA from *E. ewingii*, a closely related granulocytic *Ehrlichia* that causes disease in dogs in North America (Anderson *et al.*, 1992). The primers were able to detect, without the need of nested PCR, 10^{-3} dilution of DNA in sterile distilled water extracted from experimentally infected sheep blood, when 32 % of the neutrophils were infected, which is beyond the level of visible detectable parasitaemia by microscopy (Fig 7.7).

Sequence analysis of a 410-bp fragment amplified from the canine sample confirmed the RFLP patterns indicating a closer similarity to *E. phagocytophila* than to *E. equi* in European cases of granulocytic ehrlichiosis in hosts other than ruminants. The whole 410-bp fragment obtained from the canine sample from Utrecht is shown in Fig 7.8 and a dendrogram including the canine sample in Fig 7.9.

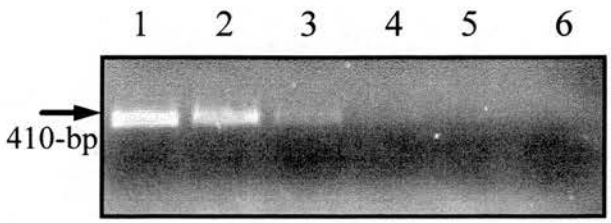
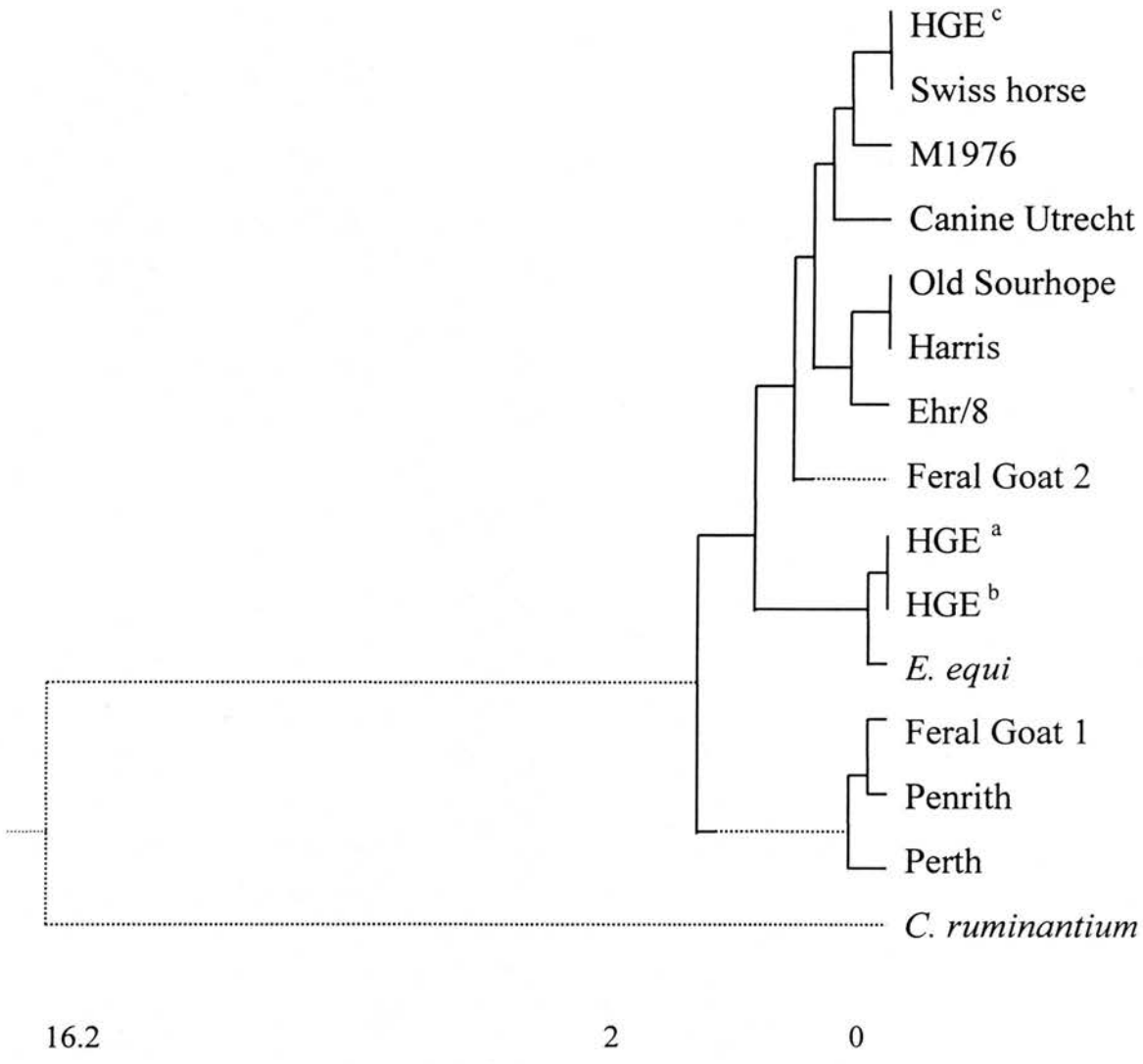


Fig 7.7 Detection threshold for primers designed to amplify a fragment of 410-bp (*groEL* gene) of granulocytic *Ehrlichia* isolates. Lane 1, neat DNA; Lane 2, 1/10 dilution in sterile distilled water of *E. phagocytophila* DNA obtained from experimentally inoculated sheep blood when 32% of its neutrophils were infected; Lane 3, 1/100 dilution; Lane 4, 1/1000 dilution; Lane 5, 1/10000 dilution; Lane 6, sterile distilled water. Primers were able to amplify 1/1000 dilutions of DNA in sterile distilled water (Lane 4).

```
                                                                    bp
TGTACTCAATAAGCTCCGTGGTGGCCTTCAAGTTGCTGCTGTAAAGGCGC                 50
CTGGTTTCGGTGACAGGAGAAAAGACATGCTTGGCGATATTGCTGTAATA                 100
GTAGGCGCTAAGTATGTAGTAAATGACGAGCTTGCTGTTAAGATGGAAGA                 150
CATCGCTCTAAGCGATCTTGGTACTGCTAAGAGCGTACGCATCACAAAAG                 200
ACACAACTACTATCATAGGTAGTGTTGATAGCAGTTCTGAAAGCATAGCT                 250
AGCAGGACTAATCAAATCAAAGCTCAGATAGAAAATTCTAGTTCTGATTA                 300
TGACAAGGAAAAGCTTAGAGAACGTTTAGCAAAGCTTTCCGGTGGTGTTG                 350
CTGTACTCAAGGTTGGTGGATCCAGCGAAGTTGAGGTGAACGAACGCAAA                 400
GACAGAGTAT                                                         410
```

Fig 7.8 *GroEL* partial sequence (410-bp) derived from a canine sample from Utrecht after PCR using primers HSP534 and HSP1324 (highlighted) specific for granulocytic *Ehrlichia*



[a] Kolbert *et al.*, 1997; GenBank accession No. U72628
[b] Sumner *et al.*, 1997; GenBank accession No. U96728
[c] Lotric-Furlan *et al.*, 1998; GenBank accession No. AF033101

Fig 7.9 Dendrogram generated with Lasergene DNAstar programme using Clustal method from an alignment of a fragment of 410-bp of *groEL* operon representing the phylogenetic relationship between several granulocytic *Ehrlichia* isolates. *Cowdria ruminantium groE* partial sequence was included for comparison

7.4 Discussion

Small subunit rRNA sequencing has been extensively used to determine phylogenetic relationship among bacteria (Woese, 1987). However, it is so highly conserved that taxonomic analysis of the granulocytic *Ehrlichia* has been constrained by its limited sequence variation within the genogroup. Species differentiation has been thus based on natural host, geographic distribution and vector.

The *groEL* gene fragment used in this, although only 813-bp in length, showed a higher number of nucleotide substitutions than occur between granulocytic isolates in 16S rDNA sequence. On average, 5 substitutions were detected in the 813-bp sequence whereas only 3 substitutions were observed in the 16S rDNA whole sequence of the same organisms (1400 bp). The similarity between agents in the genogroup appears still very high but further differences might be expected to be found after genomic analysis of less conserved genes once the identification of additional genes has been successful. Each of the sequences obtained from individual isolates contained single nucleotide changes. However, cloned material amplified via PCR typically contains mutations as expected from error rates for *Taq* polymerase (Saiki *et al.*, 1988).

Dendrogram analysis of the evolutionary relationship between granulocytic *Ehrlichia* revealed different strain associations when using two fragments of *groEL* gene of different lengths. However, in both cases two distinct clusters were formed grouping European non-ruminant isolates together but separated from granulocytic *Ehrlichia* of North American origin. It is advisable, however, to use longer fragments to resolve species or strains phylogeny.

Sequencing results in this study were in agreement with those found in previous reports from Slovenia and the United States (Sumner *et al.*, 1997; Lotric-Furlan *et al.*, 1998). In addition, we found further nucleotide differences between European isolates. Interestingly, the sequence from a patient from Slovenia (GenBank accession No. AF032101) was almost identical to the *Ehrlichia* spp. isolated from equine samples in Switzerland (GenBank accession No. U96735) and Scotland (M1976).

Sequence analysis of a blood stabilate labelled Feral Goat, although very similar to the published sequence of a sample with the same name obtained by Dr. Gordon Scott (GenBank Accession No. U96729), showed five nucleotide divergences from it. However, this stabilate was derived from a pool of blood from several goats thus it is likely that two or more strains of the pathogen were present in the same sample (Dr. Gordon Scott, personal communication). It is also possible that 16S rRNA gene has multiple versions as reported for *E. coli* (Brownlee *et al.,* 1968). Sequence discrepancies due to *Taq* polymerase incorporation errors may occur (Saiki *et al.,* 1988). Thus, all samples were sequenced twice and then compared to obtain a corrected sequence. In this study the published sequence was identified as Feral Goat1 and the new Feral Goat sequence was labelled Feral Goat2.

16S rDNA sequence analysis revealed that all granulocytic isolates from North America and Europe were practically identical at this gene level. Differences were observed, however, at informative sites. Harris strain of *E. phagocytophila*, which appears to be particularly virulent (Scott, 1982), was found identical to the HGE agent except for one nucleotide change at position 91 (Table 7.5).

A rapid method for differentiation of US and European granulocytic *Ehrlichia* isolates is presented. The North American banding pattern is predicted to occur in each of the granulocytic *Ehrlichia* agents for which GenBank database entries exist for the *groE* gene (Kolbert *et al.,* 1997; Sumner *et al.,* 1997). The European ruminant pattern also occurred for each of the entries in the database (Sumner *et al.,* 1997), but some of the ruminant isolates from the UK gave a different banding pattern that was further confirmed by HaeIII digestion. It appears that a different genotype was found in the granulocytic *Ehrlichia* of British origin that was not predicted by the sequences in the database.

The equine granulocytic *Ehrlichia* case in a Scottish horse (M1976) showed the same banding pattern that the Swiss horse (Sumner *et al.,* 1997), and was also identical to Feral Goat, Lephinmore, the two bovine isolates of Dutch and English origin, Pennine, Penrith, Ehr/8 isolated from ticks that presumably fed on deer, and R153.

According to the restriction enzyme analysis, the *E. phagocytophila*-like samples of North America are more similar to *E. equi* and the ones of European origin are more similar to *E. phagocytophila*. Although at 16S rRNA level European samples from dog and horse origin are identical to samples of HGE in the USA (Johansson *et al.*, 1995), further differences have been found at *groEL* level. 16S rRNA gene may not be a good indicator of phylogenetic relationship because of its high level of structural conservation and slow evolutionary rate (Woese, 1987). However, despite its homogeny, differences have been found between granulocytic isolates in Britain (Ogden *et al.*, 1998). A recent study using *groEL* (Petrovec *et al.*, 1999) also indicated that the granulocytic *Ehrlichia* isolates from human cases and *I. ricinus* ticks in Slovenia are more similar to *E. phagocytophila* than to *E. equi* and North American strains of HGE (Sumner *et al.*, 1997), however diversity between tick samples was also found. For closely related species it is clearly necessary to study two or more gene sequences before any conclusion can be obtained.

RFLP and sequence analysis of a sample derived from a dog from Utrecht that showed characteristic rickettsial bodies in granulocytes, also indicated a closer similarity to the sequences obtained from horses and humans in Scotland, Switzerland and Slovenia than to *E. equi* and the human cases of granulocytic ehrlichiosis in North America. Thus a pathotype specific for dogs, horses and humans appears to exist in Europe, which differs from HGE and *E. equi* in the US.

MboII showed to be a better indicator of difference than HaeIII. Although both HaeIII and MboII were able to differentiate between the same European granulocytic isolates, HaeIII analysis did not discriminate between the North American and some of the European isolates.

Sumner *et al.*, (1997) did not find any amino acid change between the *E. phagocytophila* isolates Feral Goat and Sourhope. In this study some of the isolates showed amino acid sequence variation but the differences detected between European samples did not result from the nucleotide changes that induced the two genotypes in the European isolates. It is unclear what role is played by those small differences in terms of vertebrate host association. However, they could also be due to mutations induced by errors in *Taq* polymerase nucleotide incorporation (Saiki *et*

al., 1988). It is likely that nucleotide substitutions inducing amino acid changes are related to an increased survival rate of the bacteria within a particular host thus the mutations are preserved in future generations of bacteria.

This study allowed the development of rapid methods of differentiation between granulocytic isolates from human and animals to help in rapid identification of agents from clinical cases. This typing method could be applied in the future to explore if human or animal cases other than ruminants in Europe are associated with a particular type and also in risk analysis studies with advantages of time and simplicity over sequence analysis. The role of dogs, horses, ruminants and ticks as reservoirs of human infection may also be clarified. In addition, specific primers and probes can be designed towards the intergenic spacer between *groES* and *groEL* ORFs (open reading frames) which appears to show a high degree of variation in closely related species (Sumner *et al.*, 1997).

The evidence presented in this study suggest that cases of granulocytic ehrlichiosis in hosts other than ruminants in Europe are associated with *E. phagocytophila* and differ from *E. equi* and the HGE agent isolates in North America. Sheep and other hosts in the UK may be competent reservoirs for different genotypes of granulocytic *Ehrlichia*. Isolates of *E. phagocytophila* appear to vary considerably in terms of virulence and cross-protection (Foster and Cameron, 1970; Woldehiwet and Scott, 1982b), which can be associated with phenotypic or strain variation that also appears to occur for human granulocytic isolates in the USA (Zhi *et al.*, 1998b).

In conclusion, data from this study support the evidence suggesting that cases of granulocytic ehrlichiosis in hosts other than ruminants are caused by *E. phagocytophila* in Europe. Thus the bacteria appears to be zoonotic and cross-species transmission is likely to occur in the UK and Europe as suggested by the prevalence of antibodies to the bacteria found in human samples (Sumption *et al.*, 1995; Petrovec *et al.*, 1998; Petrovec *et al.*, 1999). The observed diversity is likely to be associated

with the wide range of natural reservoirs for both granulocytic *Ehrlichia* and the vector tick.

A reorganisation of the order *Rickettsiales* appears necessary, based on sequence analysis of 16S rRNA, *groE* operon and/or other genes. More rickettsial species have to be taken into account, including those currently classified within *Bartonellaceae* and *Anaplasmataceae* families, as suggested by the inclusion of *Anaplasma marginale* within the *E. phagocytophila* cluster (see Fig 2.1, Table 2.1).

CHAPTER EIGHT, GENERAL DISCUSSION AND CONCLUSIONS

Ehrlichia species of bacteria are characterised for being fastidious obligate intracellular organisms. They are difficult to cultivate in vitro, which is required for a detailed analysis of the biology of pathogens. The next logical step will be the isolation of *E. phagocytophila* using a tick cell line, which has been successful for the closely related *Anaplasma marginale* (Munderloh *et al.,* 1996a), *E. equi* (Munderloh *et al.,* 1996b) and *Cowdria ruminantium* (Bell-Sakye *et al.,* submitted for publication). In addition, a vector cell culture system would provide an opportunity to study the relationship in vitro between the pathogen and its vector at cellular and molecular levels. On the other hand, a culture system will ensure a constant and homogeneous supply of antigen to use in serologic tests such as ELISA and Western blots. The cultivation of the HGE (Goodman *et al.,* 1996) agent has already provided the opportunity to characterise several proteins and to observe differences in the antibody responses to granulocytic *Ehrlichia* in humans depending on the isolate (Zhi *et al.,* 1998b). In this study, *E. equi* grown in tick-cell culture (Munderloh *et al.,* 1996b) was successfully used as a surrogate antigen for the detection of antibodies to granulocytic *Ehrlichia* in sheep, deer, dogs, and cats in ELISA and Western immunoblots. The results in this study indicated that it was a reliable antigen when compared to *E. phagocytophila* infected neutrophils used in IFAT. However, because it was not possible to determine the true status of infection/exposure to the pathogen for most of the samples, it is not known whether IFAT or ELISA is the test of choice for serologic diagnosis. It is advisable, however, the use of ELISA because it is quicker and allows the screening of large number of samples and then to confirm positive results, if required, using immunoblots.

Partial protection of mice against HGE challenge has been attained using bacterial lysates (Sun *et al.,* 1997), thus suggesting cell-mediated immunity may play a relevant role in protection against granulocytic *Ehrlichia.* The identification of major antigenic proteins will allow the design of a recombinant vaccine against these life-threatening emerging or rediscovered pathogens of humans and animals. A 44-KDa protein of granulocytic *Ehrlichia* appears to be a homologue of the major immunodominant protein (MAP-1) of *Cowdria ruminantium* and MSP-2 of *Anaplasma* marginale (Ijdo *et al.,* 1998; Van Vliet *et al.,* 1994; Zhi *et al.,* 1998a),

which are members of multigene families. It is not known if the existence of hypervariable regions in MAP-1 and homologues would influence the development and maintenance of protective immunity. MAP-1 of *C. ruminantium* seem to induce protection in mice (Nyika *et al.*, 1998) but the antigenic variation of MSP-2 of *A. marginale* seems to contribute to the persistence of rickettsemia in recovered animals (French *et al.*, 1998). It remains to elucidate if a recombinant 44-KDa protein can protect against infectious challenge with granulocytic *Ehrlichia*. Protective immunity is likely to be cell mediated as for *E. canis* infection (Ristic and Holland, 1993). Experiments performed by Sun *et al.*, (1997) using a murine model also indicated that antibodies provide incomplete immunity against HGE.

Mutations are fixed at very low frequencies in the 16S rRNA gene (Woese, 1987). Small differences (>0.5% for the genus *Ehrlichia*) are considered significant at the species level (Anderson *et al.*, 1991). Earlier studies using 16S rDNA sequence analysis as a molecular clock for the classification of *Ehrlichia* species indicated that the emergent cases of granulocytic ehrlichiosis in horses, dogs and humans of Europe were almost identical to the HGE agent in North America and differed from *E. phagocytophila* (Chen *et al.*, 1994; Johansson *et al.*, 1995). Sequence analysis of a different gene, the *groE* operon, which is also very well conserved, has revealed a closer relationship between several *E. phagocytophila* blood stabilates isolated by Dr. Gordon Scott and the non-ruminant cases of granulocytic ehrlichiosis in the UK and Europe. This evidence suggests that cross-species transmission of *E. phagocytophila* to humans, dogs and horses in Europe is possible and indicates that the bacteria is potentially zoonotic as observed by the production of similar clinical responses in experimental infections between species (Madigan *et al.*, 1995). Nucleotide differences were found however between ruminant and equine isolates suggesting that *E. phagocytophila* strains differ in their vertebrate host specificity. Thus, it is suggested that classification of *Ehrlichia* and other closely related pathogens should be based on phylogenetic analysis of two or more genes rather than host preference.

In this study, roe deer have been identified as competent reservoirs for *E. phagocytophila*. *Ehrlichia phagocytophila groE* gene was partially amplified by PCR

in blood and spleen and antibodies were detected in 58% of roe deer samples. Other species such as dogs, cats and horses also appeared to show immune reactions to the bacteria in the UK thus suggesting that exposure is common in domestic and wild animals. The non-specific symptoms and the uneventful recovery that characterises the disease if uncomplicated are probably responsible for the delay in diagnosis and the low numbers of cases reported. The role of horses, dogs and cats in the epidemiology of the disease is to be determined. However, the evidence suggests that pet animals are aberrant or 'dead end' hosts for the bacteria like humans because they are not competent maintenance hosts for ticks. This was confirmed by the higher prevalence of antibodies to *E .phagocytophila* in working dogs and horses from rural areas of Britain. Risks of acquiring the disease in humans appears to be low in the UK, recent studies have detected low prevalence of antibodies to tick-borne zoonoses in farmers of England (Thomas *et al.,* 1998). Higher risk can be expected when humans are exposed to ticks in woodland areas where high densities of both deer and tick populations may be found and also higher prevalences of infection in ticks appear to occur as demonstrated in this study. In addition, despite two peaks of tick activity in spring-summer and autumn appear to occur in upland areas of the UK, ticks in woodlands may quest continuously with a peak of activity in the summer as observed in the numbers of ticks found on roe deer legs in chapter four and in different studies on pheasants and rodents (Craine *et al.,* 1995). It is likely that deer abundance is indirectly related to the intensity of *Ixodes*-borne zoonoses. Despite they are not competent reservoirs for some pathogens such as *Borrelia* spirochetes (Jaenson and Talleklint, 1992), deer are a major blood source for all stages of ticks. Increasing numbers of cases of Lyme disease in Sweden have been linked to an increase in the roe deer population (Jaenson, 1991). Paradoxically, abundant numbers of deer or other incompetent vertebrate species may also reduce the proportion of infective ticks (dilution effect) by avoiding large numbers of ticks feeding on more efficient reservoirs for diseases such as small mammals.

When ticks feed as larvae they maintain the infection even when feeding as nymphs on refractory hosts. Higher percentages of infected adults should thus be expected. It was found, however, that the rate of infection in adult ticks was lower

than in nymphs. Little is known about the role of co-feeding in ticks for the amplification of *Ehrlichia* infection between ticks. Co-feeding transmission has been found to occur experimentally for louping-ill virus in ticks feeding on mountain hares but trials using red deer as hosts for *Ixodes ricinus* ticks and the virus have been hitherto unsuccessful (Jones *et al.*, 1997). The low prevalence of infection found in field collected nymphs, and particularly adults, suggests that co-feeding is not an important mechanism for the maintenance of *E. phagocytophila* infection in nature. On the other hand, *Ehrlichia phagocytophila* could not survive well in adult ticks or perhaps the survival of infected adult ticks is reduced by the infection. It is known that bacterial and nematode infection of *Ixodes* ticks have pathogenic effects on them (Kocan *et al.*, 1998).

Transmission experiments using *Ixodes ricinus* larvae feeding on experimentally infected sheep in order to determine if there was a difference in the transmission rate when ticks were feeding during patent parasitaemia and latent infection were unsuccessful. A combination of the pathogenic effect of *Ehrlichia* infection within the ticks together with the development of acquired resistance to tick feeding in sheep hindered the progress of the experiment. Very few ticks were recovered alive and when examined none of them showed evidence of *Ehrlichia* infection. Different isolates of *E. phagocytophila* may have different vertebrate host and tick preferences. The stabilate used to infect sheep was derived from ticks that were most likely feeding on deer. Thus, although experimental infection was attained in sheep as confirmed by means of serology and PCR, it is possible that the bacteria were not well adapted to sheep and therefore were not infective for ticks. On the other hand, acquired resistance to tick feeding in sheep (Abdul-Amir and Gray, 1987) may be a mechanism to avoid infections with an immunosuppressive bacteria like *E. phagocytophila* that combined with other conditions such as tick-pyemia produces many losses and ill-growth in young lambs which are fully naïve to tick infestations.

Ehrlichia phagocytophila and *C. ruminantium* are the closest known relatives to mitochondrial organelles (Weisburg *et al.*, 1989; Viale and Arakaki, 1994), which evolved from free-living organisms to endosymbionts within eukariotyc cells.

Immunoblot studies, using beef heart mitochondria as antigen, suggested that mitochondria and *Ehrlichia* share antigenic epitopes and that some of the anti-ehrlichial antibodies may be anti-mitochondrial as demonstrated by the recognition of mitochondrial proteins in serum that contained antibodies to either *E. phagocytophila* or *C. ruminantium*. It appears essential to further characterise those similar antigenic epitopes in order to develop a vaccine against *Ehrlichia* that does not interfere with the host immunity, specially if repeated boosters are necessary. It is known that repeated bacterial exposure can lead to autoimmune responses like in primary biliary cirrhosis (PBC), frequently associated with recurrent urinary infection. Mitochondrial proteins could be further used as surrogate antigens for *Ehrlichia* to identify major sharing antigenic components of *Ehrlichia* and to help in our understanding of the immune responses towards the bacteria.

CONCLUSIONS

(i) Human, equine and canine granulocytic *Ehrlichia* isolates from Europe and the United States appeared hitherto to be almost identical at 16S rDNA sequence level. This study has revealed, through *groE* operon gene analysis, closer relationship between ruminant and non-ruminant samples of European origin than to non-ruminant cases of granulocytic ehrlichiosis in the US. The data suggest that *E. phagocytophila* causes a tick-borne zoonosis and cross-species transmission is likely to occur in Europe. The closer similarity of human to dog and horse isolates of *E. phagocytophila* in Europe suggests there may be a higher risk for infection in humans associated with pet contact. Thus, public education appears essential to reduce the number of ticks attached to both humans and domestic animals.

(ii) Emerging cases of granulocytic ehrlichiosis seem to be associated with an increased level of awareness and development of better diagnostic methods, including molecular techniques, thus allowing the characterisation of non-cultivable pathogens such as *E. phagocytophila*. A re-classification of the bacteria based on genetic evidence rather than host specificity is recommended. Further studies are required to elucidate the relationship between the taxonomy of granulocytic *Ehrlichia* and its antigenic heterogeneity, virulence and vertebrate host relationship. Different forms of granulocytic *Ehrlichia* in different environments may occur because of a combination of mammalian hosts and tick populations factors through which the infection is passed.

(iii) Dogs, cats, horses and roe deer samples from the UK showed reactivity to *E. phagocytophila* antigens indicating that exposure to the pathogen is common. Higher seroprevalences were observed in domestic dogs from rural areas and in wild roe deer populations associated with higher densities of *I. ricinus* tick infestations. PCR assays with specific primers may be used for detecting the organisms in potential animal reservoirs and tick vectors and as a means of early diagnosis.

(iv) Variable but consistently low prevalences of infection were found in field collected ticks as confirmed by several methods such as Feulgen and IFA staining of

salivary glands and PCR. However, because high tick infestations are usually found in wild hosts such as roe deer as demonstrated in this study, it is still possible for the disease to be maintained in nature. Risk of infection for humans and pet animals appears to be low because they do not normally support high tick infestations and not every tick bite is infectious. The lack of transovarial transmission and the low levels of infection found in *Ixodes* suggest infected ticks are highly efficient vectors. *Ehrlichia phagocytophila* ensures its survival in nature by having a wide range of susceptible vertebrate hosts in which the bacteria may induce persistent infections.

(v) Data obtained in this study give strong evidence to support the implication of roe deer as a natural host for *E. phagocytophila*. However, transmission experiments are required to confirm its role as a competent reservoir for the bacteria. Rodents and others species of deer should also be studied in order to clarify the endemic cycles of the disease in nature.

(vi) Distinct antigenic and immunogenic properties are expected to be found between different isolates of *E. phagocytophila* as supported by cross-protection trials in ruminants. The observed DNA sequence polymorphism between human and animal isolates is likely to be associated with underlying antigenic differences. Cultivation of the bacteria in continuous cell lines is essential for a better characterisation of the biological properties of the pathogen and the identification of major antigenic components that will help in the development of vaccines.

(vii) Mitochondria can be used as a surrogate antigen for the characterisation of major antigenic epitopes of *Ehrlichia* species as demonstrated by the presence of antibodies reacting to mitochondrial proteins in immunoblots using experimentally inoculated sheep and goat sera. An understanding of the nature of the immune response towards *Ehrlichia* will help in the development of vaccines towards these life threatening pathogens.

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APPENDICES

COMMONLY USED BUFFERS

A. Buffers for ELISA, IFAT and Feulgen

Bicarbonate buffer 0.1 M, pH 9.0-9.2

Stock solution A		
Anhydrous sodium carbonate	0.2 M	21.2 g in 1 l of dH_2O
Stock solution B		
Sodium hydrogen carbonate	0.2 M	16.8 g in 1 l of dH_2O

Mix 5 ml of solution A with 45 ml of solution B and add 50 ml of dH2O for 100 ml bicarbonate buffer solution 0.1 M, pH 9.0-9.2

Phosphate Buffered Saline (PBS) solution for IFAT pH 8.0

Na_2HPO_4 (anhydrous)	0.1 M	13.48 g
NaH_2PO_4	5 mM	0.78 g
NaCl	0.07 M	4.25 g
Distilled water		for 1 l solution

Dilute 6:4 in distilled water for use

PBS Dulbecco-A pH 7.3

NaCl	0.1 M	8 g
KCl	3 mM	0.2 g
Na_2HPO_4 (anhydrous)	8 mM	1.15 g
KH_2PO_4	1.5 mM	0.2 g
Distilled water		for 1 l solution

Carbonate-bicarbonate buffer, pH 9.6, 0.05 M (ELISA coating buffer)

Na_2CO_3	15 mM	1.59 g
$NaHCO_3$	35 mM	2.93 g
Distilled water		for 1 l solution

or

1 capsule (Sigma) in 100 ml of deionised distilled water

Blocking buffer

Normal rabbit serum	4%
PBS	1x
Tween 20	0.1%

Phosphate-citrate buffer, pH 5.0, 0.05 M, 0.014% H_2O_2

1 tablet (phosphate-citrate buffer with urea hydrogen peroxide tablets, Sigma) in 100 ml of deionised distilled water

TMB Microwell peroxidase substrate, 1 component

Kirkegaard & Perry Laboratories, Maryland, USA

Lysis buffer (for *E. equi* antigen for ELISA)

NP-40 (Nonidet P-40)	0.5%
Dexycholic acid (sodium salt) in TEN	0.5%

Protease inhibitor cocktail (cØmplete™, Mini EDTA-free, Boehringer Mannheim)

7x stock solution	1 tablet/ 1.5 ml dH_2O,

TEN

Tris pH 7.4	50 mM	6.05g
NaCl	150 mM	8.77g
EDTA	2 mM	0.74g

TBS (Tris buffered saline) pH 7.6

Tris-base	20 mM	2.42 g
NaCl	137 mM	8 g
HCl	1 M	3.8 ml
Distilled water		for 1 l solution

PBST (0.1%)

PBS (1x concentrated)	1000 ml
Tween 20	1 ml

Growth Medium for *Cowdria ruminantium*

RPMI medium (Sigma) supplemented with	
Foetal bovine serum	10%
Tryptose phosphate broth	10%
HEPES buffer	20 mM
L-glutamine	2 mM
Penicillin	100 µg/ml
Streptomycin	100 µg/ml
Fungizone	2.5 µg/ml

Schiff's reagent for Feulgen staining (for 200 ml solution)

Pararosaniline chloride	1g
Potassium metabisulphite	2 g
Hydrochloric acid	2 ml
Powdered charcoal	2 g

B. Buffers used for Western blot

5% Blocking buffer (SDS-PAGE)

Commercial skimmed milk	5 g
PBST	100 ml

Bjerrum and Schaefer-Nielsen transfer buffer

Tris-base	48 mM	5.82 g
Glycine	39 mM	2.93 g
Methanol	20 %	
SDS	1.3 mM	0.0375 %
Distilled water	for 1 l solution	

Reagents and gel preparation for SDS-PAGE slab gels (Laemmli buffer system)

Stock solutions

Acrylamide/bis	40%	
Tris-HCl, pH 8.8	1.5 M	18.15 g Tris base (adjust pH to 8.8 with 1 N HCl. Make 100 ml with distilled water)
Tris-HCl, pH 6.8	0.5 M	6 g Tris base (adjust to pH 6.8 with 1N HCl. Make 100 ml with distilled water)
SDS	10%	
Ammonium persulphate (APS)	10% (100 mg/ml)	

Separating gels (10%)

Distilled water	4.05 ml
1.5 M Tris-HCl pH 8.8	2.5 ml
10% SDS	100 µl
40% Acrylamide	3 ml
TEMED	5 µl (0.5 µl/ml)
10% Ammonium persulphate	50 µl (5 µl/ml)

Stacking gel (4%)

Distilled water	6.1 ml
0.5 M Tris-HCl pH 6.8	2.5 ml
10% SDS	100 µl
40% Acrylamide	1 ml
TEMED	10 µl
10% Ammonium persulphate	50 µl (5 µl/ml)

Sample buffer (SDS reducing buffer), 2x stock

Distilled water	8.2 ml
0.5 M Tris-HCl pH 6.8	5 ml
Glycerol	4 ml
10% SDS	0.8 g
2-β-mercaptoethanol	2 ml
0.05% (w/v) bromophenol blue	spatule tipful

Electrode running buffer (5x), pH 8.3

Tris-base	0.1 M	15 g
Glycine	1 M	72 g
SDS	17 mM	5 g
Distilled water		for 1 l solution

Reagents for phosphate assay

Reagent A	0.34 M sodium acetate 2 M acetic acid 0.25% $CuSO_4.5H_2O$
Reagent B	5% Ammonium molybdate

Reagent C	2% p-methylaminophenol sulphate (elon) 5% Na_2SO_3
Standard Pi	0.5 mM KH_2PO_4

ATPase assay mix

ATP	0.1 M, pH 7
$MgSO_4$	0.1 M
Hepes-NaOH	1 M, pH 7.4
Distilled water	

C. Buffers used for PCR, electrophoresis of PCR products and cloning

DNA loading buffer (blue juice)

EDTA	75 mM
Bromophenol blue	0.1 %
Glycerol or sucrose	25 %
MilliQ water	

TBE 10x

Tris-base	0.9 M	108 g
Boric acid	0.9 M	55 g
EDTA pH 8.0	0.5 M	186.1 g
Distilled water		up to 1 l solution

Loening E buffer 5x

Tris-base	0.2 M	43.4 g
NaH_2PO_4	0.2 M	47 g
EDTA	6 mM	3.7 g
		for 2 litres solution

7.5 % Polyacrylamide gels for DNA electrophoresis

40%Acrylamide	2 ml
Loening E buffer (5x)	2 ml
TEMED	10 µl
10 % Amonium persulphate	70 µl
Distilled water	6 ml

Silver nitrate staining of polyacrylamide DNA gels

Fixative	Ethanol	10%	
	Glacial acetic acid	0.5%	
Stain	$AgNO_3$	11 mM	0.19 g
Developer	NaOH	0.75 M	3 g
	Formaldehyde	0.75 ml	
Stop solution	Na_2CO_3	0.07 M	0.75 g

Nucleotides (dNTP's)

Stock solution	2 mM

10 µl of each nucleotide in 460 µl of sterile MilliQ water

Luria Bertani Medium (pH 7.0)

Tryptone	1%
Yeast Extract	0.5%
Na Cl	1%

Luria Bertani agar

As for Luria Bertani Medium but adding 15g/l of agar before autoclaving

SOC Medium

Tryptone	2%
Yeast Extract	0.5%
Na Cl	10 mM
K Cl	2.5 mM
Mg SO_4	10 mM
Glucose	20 mM

D. Buffers for Southern blot/dot blot

Depurination solution

HCl	0.25 M

Denaturation solution

NaOH	0.5 N
NaCl	1.5 M

Neutralisation solution

Tris-HCl, pH 7.5	0.5 M
Na Cl	3 M

SSC stock solution (20x), pH 7.0, autoclaved

NaCl	3 M
Sodium citrate	0.3 M

N-lauroylsarcosine stock solution

10% (w/v) in H_2O

SDS stock solution

10% (w/v) in H_2O

Blocking reagent stock solution, autoclaved

Blocking reagent included with DIG DNA detection kit dissolved in maleic acid buffer to a final concentration of 10% (w/v)

Standard hybridisation buffer

SSC	5 x
N-lauroylsarcosine	0.1%
SDS	0.02%
Blocking reagent	1%

Maleic acid buffer (Buffer 1), pH 7.5 adjusted with solid NaOH, autoclaved

Maleic acid	0.1 M
NaCl	0.15 M

Washing buffer

Buffer 1	50 ml
Tween 20	0.3% (v/v)

Buffer 2

Blocking stock solution diluted 1/10 in Buffer 1

Buffer 3, pH 9.5

Tris-HCl	0.1 M
NaCl	0.1 M
$MgCl_2$	0.05 M

Buffer 4, pH 8.0

Tris-HCl	10 mM
EDTA	1 mM

Anti-DIG-AP conjugate

Polyclonal sheep anti-digoxigenin Fab-fragments conjugated with alkaline phosphatase (750 U/ml)

NBT/BCIP stock solution

5-bromo-4-chloro-3-indolyl phosphate (BCIP)	
nitroblue tetrazolium salt (NBT)	
DMSO	67%

Appendix 3.1 Summary of primers used in the study

Target	Size of DNA amplicon (Ref.)	Designation	Nucleotide sequence (5' -3')
16S rDNA	151-bp (Munderloh *et al.*, 1996b)	856	GAATTGACGGGGACCCGCACAAGCG
		1154	AAGGGCCGTGCTGACTTGACATCATC
		GER3	TAGATCCTTCTTAACGGAAGGGCG
		GER4	AAGTGCCCGGCTTAACCCGCTGGC
groE	813-bp (Kolbert *et al.*, 1997)	HSP354	CGYCAGTGGGCTGGTAATGAA
		HSP2165	CCATACCWCCCATGCCTC
		HSP961	GAAGAAATTGCHCAAGTWGC
		HSP1754a	TTCTTCAACAGCWGCTCTAG
	410-bp (Alberdi, unpublished)	HSP534	TGTACTCAATAAGCTCCGTGGTG
		HSP1326	CTACTCTGTCTTTGCGTTCCTTCA
Cytochrome b	638-bp (Kirstein and Gray, 1996)	cytb1	CCATGAGGACAAATATCATTCTG
		cytb3	GGGTGTTCDATGGYTGBCCYCC
	134-bp	2cytb1	GGMTTYTCAGTAGACAAAGC
		2cytb6	AWTCCTGTKGGGTTRTT
	95-bp	3cytb1	ACCYTNACCYCGATTYTTCGC
		2cytb5	GAKCCTGTYTCGTGGAGGA

W = A or T; B = G, T or C; Y = C or T; D = *G*, A, or T; M = A or C; K = G or T; R = A or G; N = A, G, C, or T

Appendix 3.2. Data from experimentally inoculated lambs

	Temperature							
Day after experimental inoculation	**Lamb number**				Mean Temperature	Median	Standard Deviation	Standard Error
	2228	**2342**	**2204**	**2328**				
0								
1								
2								
3	40.8	41.2	41.1	41.7	41.2	41.15	0.32	0.16
4								
5	42	42	42.4	41.6	42	42	0.28	0.14
6	41.6	41.8	41.6	41.5	41.625	41.6	0.11	0.05
7	41.3	41.5	41.4	41	41.3	41.35	0.19	0.09
8	40.9	41	41.8	40.1	40.95	40.95	0.60	0.30
9	40.7	40.9	41.4	40.3	40.825	40.8	0.40	0.20
10	40.5	40.4	40.5	39.7	40.275	40.45	0.33	0.17
11	40.5	40.3	40.6	39.9	40.325	40.4	0.27	0.13
12	40.6	40.3	40.6	40	40.375	40.45	0.25	0.12
13	40.3	39.7	40.6	40	40.15	40.15	0.34	0.17
14	40.1	40.6	40	39.6	40.075	40.05	0.36	0.18
15	40	40.1	40.2	40.1	40.1	40.1	0.07	0.04
16	41.2	40	40.6	40.1	40.475	40.35	0.48	0.24
17	41.7	40.4	40.3	40	40.6	40.35	0.65	0.33
18	40.4	40.2	40	40	40.15	40.1	0.17	0.08
19	39.9	40.2	39.9	40.6	40.15	40.05	0.29	0.14
20	39.9	40.2	40.6	41.3	40.5	40.4	0.52	0.26
21	40.1	40.2	40.3	40	40.15	40.15	0.11	0.06
22	39.6	39.9	39.7	39.6	39.7	39.65	0.12	0.06
23								
24								
25	39.5	39.9	39.6	39.6	39.65	39.6	0.15	0.07
26	39.6	39.7	39.6	39.4	39.575	39.6	0.11	0.05
27	40.1	40.1	40.2	40	40.1	40.1	0.07	0.04
28	39.6	39.7	39.6	39.6	39.625	39.6	0.04	0.02
29	39.8	39.6	39.9	39.7	39.75	39.75	0.11	0.06
30	39.7	39.5	39.9	39.6	39.675	39.65	0.15	0.07
31	39.6	39.6	39.9	39.9	39.75	39.75	0.15	0.08
32	39.8	39.6	39.8	40.1	39.825	39.8	0.18	0.09
33	39.7	39.8	40	39.8	39.825	39.8	0.11	0.05
34	39.8	40	40.1	39.8	39.925	39.9	0.13	0.06
35	39.6	39.7	39.9	39.7	39.725	39.7	0.11	0.05

Appendix 3.2 Cont.

	% Infected neutrophils							
Day after experimental inoculation	**Lamb number**				Mean	Median	Standard Deviation	Standard Error
	2228	**2342**	**2204**	**2328**				
0								
1								
2								
3	0	2	3	2	1.75	2	1.26	0.63
4								
5	16	25	28	20	22.25	22.5	5.32	2.66
6	31	30	26	33	30.00	30.5	2.94	1.47
7		21	33	32	28.67	32	6.66	3.33
8	11	18	29	21	19.75	19.5	7.46	3.73
9	12	17	26	16	17.75	16.5	5.91	2.95
10	5	9	13	8	8.75	8.5	3.30	1.65
11								
12								
13	0	1	4	1	1.50	1	1.73	0.87
14								
15								
16								
17	2	0	0	0	0.50	0	1	0.50
18								
19								
20	0	0	0	0	0	0	0	0
21	0	0	0	0	0	0	0	0
22	0	0	0	0	0	0	0	0
23								
24								
25								
26								
27								
28	0	0	0	0	0	0	0	0
29								
30								
31	0	0	0	0	0	0	0	0
32								
33								
34	0	0	0	0	0	0	0	0

Appendix 4.1 Summary of details from roe deer legs separated according to the sampling site

My No.	Reference	No. ticks on legs			PCR		IFAT	Sex	Age
		Larvae	Nymphs	Adults	Blood	Spleen	(titre)		
Borders									
1	KU230295						-	M	C
2	KU250295						+ (400)	F	C
3	KU250295						-	F	C
4	KU310395						-	F	C
5	KU211095						-	M	A
6	KU211095						+ (800)	F	A
7	KU041195						+ (6400)	M	A
8	KU130196						-	F	A
9	KU140196						+ (100)	M	A
10	KU240296						-	F	A
11	KU290696						-	M	A
12	KU280297						-	M	C
13	KU030497						-	M	A
14	KU030497						-	M	A
15	KU261197				-		+ (200)		
16	KU041297				-		+ (100)		
17	KU131297				-	-	-		
18	KU150198				-	-	-		
19	KU120298				-	+	-	F	C
20	KU120298				-	-	-	F	C
21	KU190298				-	-	-	M	C
22	KU260298				-	-	-	F	A
23	KU070398				-	-	-	F	A
24	KU120398	0	0	0	-	-	-	F	A
42	KU120498	0	0	0	-	-	+ (100)	M	A
58	KU180498	0	0	0	-	-	-	M	A
59	KU121294						-	F	C
60	KU261294						-	F	A
61	KU120195						-	F	A
62	KU120195						-	F	C
63	KU180595						-	M	A
64	KU211096						-	F	A
67	KU280598	5	0	0	-	-	-	M	C
91	KU211098	0	0	0	-	-	+ (100)	F	A
92	KU211098	0	0	0	-	-	+ (100)	F	C
98	KU011198	0	0	0	-	-	-	M	C
99	KU011198	1	0	0	-	-	-	M	C
100	KU011198	1	0	0	-	-	+ (100)	F	A

My No.	Reference	No. ticks on legs			PCR		IFAT	Sex	Age
		Larvae	Nymphs	Adults	Blood	Spleen	(titre)		
Kyloe									
37	JB280398	92	99	0	+	+	+ (3200)	F	A
38	JB280398	65	27	0	-	+	-	M	A
39	JB110498	4	16	0	+	-	+ (400)	F	C
40	JB110498	18	15	0	-	-	+ (3200)	M	
41	JB110498	5	4	0	+	+	-	M	
45	JB260498	95	26	0	+	-	+ (1600)	M	
46	JB260498	94	29	0	+	-	-	F	C
47	JB260498	39	30	0	+	-		F	A
50	JB010598	454	95	0	-	-		M	A
54	JB020598	60	21	1	-	-	-	M	C
66	JB240598				-	-	+ (1600)	M	A
76	JB110798				-	-	+ (400)	M	C
80	JB270798				+	+	+ (100)	M	A
81	JB270798				+	-	+ (400)	M	A
82	JB310798				-	-	-	M	A
83	JB310798				-	-	-	M	A
84	JB310798				-	-	-	M	A
85	JB020898				-	-	+ (3200)	M	A
95	JB311098	8	4	0	+	+	+ (3200)	F	A
96	JB011198	2	0	0	-	-	+ (1600)	F	A
105	JB061198	0	1	0					
106	JB061198	8	3	0					
107	JB071198								
108	JB141198	7	7	0					
Auchtertyre									
26	AY160398	0	0	0	-	-	+ (3200)	F	C
27	AY170398	0	1	0	-	-	+ (6400)	F	C
28	AY210398	0	3	0	+	+	+ (3200)	F	C
29	AY210398	1	0	0	+	-	+ (3200)	F	A
30	AY230398	9	7	0	+	+	+ (3200)	M	C
36	AY250398	5	3	0	-	+	+ (1600)	F	A
43	AY130498	3	3	0	+	-	+ (3200)	M	A
44	AY240498	57	13	0	+	-	-	M	A
52	AY300498	23	10	0	+	-	+ (3200)	M	A
55	AY060598	53	18	1	+	+	-	M	C
68	AY270598				+	-	+ (12800)	M	A
70	AY040698				+	+	+ (3200)	M	A
71	AY040698				+	-	+ (6400)	M	A
73	AY130698				+	+	+ (400)	M	A
89	AY061098	1	0	0	+	+	+ (6400)	M	A
93	AY231098	0	1	0	+	+	+ (3200)	F	A
94	AY261098	3	2	0	-	+	+ (400)	F	A
102	AY021198	3	1	0	+	+	-	F	A

My No.	Reference	No. ticks on legs			PCR		IFAT	Sex	Age
		Larvae	Nymphs	Adults	Blood	Spleen	(titre)		
Euston									
48	HR280498	3	9	0	+	-	+ (3200)	M	C
49	HR300498	16	8	0	-	-	+ (400)	M	A
51	HR300498	22	2	0	-	-	+ (12800)	M	A
53	HR020598	13	23	0	-	-	+ (3200)	M	A
56	HR080598	0	5	0	-	-	+ (3200)	M	A
57	HR160598	215	34	0	-	-	+ (12800)	M	A
65	HR230598	12	17	0	+	-	+ (1600)	M	C
69	HR300598	27	20	0	+	-	+ (400)	M	C
86	HR020898	300	10	0	+	+	+ (3200)	M	A
87	HR030898	636	22	0	+	+	-	M	A
101	HR031198	1	3	0	+	+	+ (12800)	F	A
103	HR031198	0	0	0	-	-	+ (1600)	F	A
104	HR031198	0	1	0	-	-	+ (3200)	F	A
109	HR161198	1	3	0					
110	HR161198	1	0	0					
111	HR161198	3	3	0					
112	HR191198	0	1	0					
Moncreiffe									
31	HR230398	9	4	0	-	+	+ (400)	F	A
32	HR230398	30	30	0	+	+	+ (1600)	F	A
33	HR230398	33	24	0	+	+	+ (3200)	F	C
34	HR240398	2	3	0	-	+	+ (3200)	F	A
35	HR240398	3	5	0	+	+	+ (3200)	F	A
77	HR130798	44	7	0	-	+	+ (400)	M	A
78	HR170798	39	15	1	+	-	+ (3200)	M	A
79	HR170798	48	25	0	+	+	+ (3200)	M	A
Kirkhouse									
25	CM150398	14	0	0	-	+	+ (3200)	F	A
75	CM070798	48	8	0	-	-	+ (3200)	M	C
88	SB150898	1	0	0	-	-	+ (400)	M	A
Balblair									
74	CS230698	29	6	0	-	-	-	M	A
Dundas									
72	JR160698	4	0	0	-	-	-	M	A
97	JR311098	0	0	0	-	-	-	F	A
Wytham									
90	HR151098	1	0	0	-	-	-	M	A

Blank spaces indicate no data were available

M Male A Adult

F Female C Calf

Appendix 4.2 Comparison on the number of ticks collected using blanket drags in two different forests Nether Stewarton (Borders, site 4) and Kyloe (site 6) which showed a low and high prevalence of infection to *E. phagocytophila*, respectively, in roe deer.

	Instar of tick				
	F	L	N	M	F
No. drags	Borders		Kyloe		
1	0	0	6	2	0
2	0	0	6	0	0
3	0	0	10	3	1
4	0	0	2	3	1
5	0	20	5	0	0
6	0	0	1	0	0
7	0	0	0	1	0
8	0	0	6	0	0
9	0	0	6	2	0
10	0	0	4	0	0
11	0	0	2	0	0
12	0	0	5	0	0
13	0	0	2	0	1
14	0	0	0	0	1
15	0	20	0	0	1
16	0	10	3	0	0
17	0	0	2	0	0
18	0	0	0	0	0
19	0	0	1	0	0
20	0	5	2	0	0
21	0	50	2	0	0
22	0	0	4	0	0
23	0	10	1	0	0
24	0	5	4	0	0
25	0	0	5	0	0
26	0	0	3	0	0
27	1	0	2	0	0
28	0	0	4	0	0
29	0	0	2	0	0

L Larvae
N Nymph
M Male
F Female

Appendix 4.2 Cont.

	Instar of tick				
	F	L	N	M	F
No. drags	Borders		Kyloe		
30	0	0	9	0	0
31	0	0	1	0	0
32	0	0	9	6	2
33	0	0	4	2	0
34	0	0	4	3	2
35	0	0	3	1	2
36	0	0	3	6	0
37	0	0	3	0	1
38	0	0	3	1	1
39	0	0	0	0	1
40	0	0	1	0	0
41	-	0	2	0	0
42	-	0	7	1	2
43	-	0	3	0	1
44	-	0	4	2	4
45	-	0	5	1	0
Total	1	120	151	34	21

L Larvae
N Nymph
M Male
F Female

Appendix 4.3 Roe deer cytochrome b partial DNA sequence, 638-bp

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CCATGAGGACAAATATCATTCTGAGGAGCAACAGTTATTACCAATCTCCT  50
CTCAGCAATTCCATATATCGGTACAAACCTAGTTGAATGAATCTGAGGGG 100
GCTTTTCAGTAGACAAAGCAACCCTGACCCGATTTTTCGCTTTCCACTTT 150
ATTCTCCCATTTATCATTGCAGCACTTGCTATAGTCCATTTACTTTTCCT 200
CCACGAAACAGGATCAAACAACCCGACAGGAATCCCATCAAACGCGGACA 250
AAATTCCATTTCACCCCTACTACACCATTAAAGATATCCTAGGAATTCTA 300
CTCTTAATTCTTTCCCTAATATTACTAGTCCTATTCGCACCAGACCTGCT 350
TGGGGACCCAGATAACTACACACCAGCAAATCCACTTAACACACCCCCTC 400
ACATTAAACCAGAATGGTACTTCTTATTTGCATACGCAATCCTACGATCT 450
ATTCCTAACAAACTAGGAGGAGTACTAGCCCTAATCTCATCAATCCTAAT 500
CTTGATCCTTATACCCCTCCTCCATACATCTAAACAACGCAGTATAATGT 550
TCCGGCCATTTAGTCAATGCTTATTCTGAATCCTAGTAGCTGACCTATTA 600
ACACTAACATGAATTGGGGGCCAGCCAGTCGAACACCC             638
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Appendix 5.1 IFAT reverse dilution of titres at several times after infection from sheep experimentally inoculated with *E. phagocytophila* (Kindly provided by E. Paxton)

	IFAT titre											
Sheep No.	928	929	930	931	932	933	934	935	936	937	938	939
Day 0	0	0	0	0	0	0			0	0	0	0
4 days	0	0	0	0					0	0	0	0
1*	0	0	0	0	0	0	0	0	0	0	0	0
2	3160	3160	10000	10000	10000	10000		10000	10000	10000	3160	3160
3	10000	3160	3160	10000		10000		10000	3160	10000	3160	10000
4	10000			3160					10000	3160	3160	3160
8	3160	1000	3160	3160					3160	3160	3160	3160
12	1000	1000	1000	3160					3160	3160	3160	3160
16	3160	3160	1000						3160	1000		
20	1000	316							1000	316		
24	316	316	1000							316		
28			1000									
32			316									
36			316									

* weeks after experimental inoculation

Appendix 5.2 ELISA using 12 different peptides against samples with antibodies to *E. phagocytophila*, *C. ruminantium* and *E. canis*

			Peptide, % reaction					
	Host	Sample	13HGE	61HGE	82HGE	89HGE	61cha	82cha
Negative	Sheep	*E. phagocytophila*	22.78	15.94	7.77	5.84	13.63	5.53
	Sheep	*E. phagocytophila*	1.65	2.09	2.17	6.08	0.52	5.65
	Goat	*C. ruminantium*	15.89	15.28	5.61	0.00	10.54	4.84
	Dog	*E. canis*	17.50	-9.88	-9.02	-9.18	-8.64	-5.74
	Dog	*E. phagocytophila*	8.91	-12.11	-11.08	-7.59	-11.36	-5.43
Positive	Sheep	*E. phagocytophila*	33.40	50.35	11.41	-17.36	56.43	7.32
	Sheep	*E. phagocytophila*	22.42	10.31	11.00	-3.73	15.57	4.72
	Goat	*C. ruminantium*	0.95	7.17	33.25	-18.83	19.78	20.98
	Goat	C. *ruminantium*	28.96	40.53	28.35	4.61	27.58	14.02
	Dog	*E. canis*	25.46	1.36	-3.91	5.13	1.69	6.04
	Dog	*E. canis*	62.05	10.42	2.13	17.35	12.41	24.94
	Dog	*E. phagocytophila*	17.98	0.64	3.78	3.06	1.45	11.01
	Dog	*E. phagocytophila*	4.24	-3.50	-1.72	0.50	-3.36	5.02
			89cha	13Cow	61Cow	82Cow	89Cow	61canis
Negative	Sheep	*E. phagocytophila*	5.15	16.69	7.74	7.93	11.27	10.45
	Sheep	*E. phagocytophila*	2.00	6.69	-0.96	7.21	7.82	-1.65
	Goat	*C. ruminantium*	0.35	17.70	10.45	2.85	2.68	6.91
	Dog	*E. canis*	-10.95	9.11	-11.06	-10.25	1.52	-11.44
	Dog	*E. phagocytophila*	-10.18	-4.25	-10.82	-10.62	2.92	-12.32
Positive	Sheep	*E. phagocytophila*	3.21	6.40	23.08	8.95	6.49	16.21
	Sheep	*E. phagocytophila*	-10.41	45.39	59.13	13.15	-16.75	54.17
	Goat	*C. ruminantium*	2.42	-1.38	18.83	22.80	-17.62	23.83
	Goat	C. *ruminantium*	6.85	14.36	57.54	20.93	16.52	35.61
	Dog	*E. canis*	-1.38	6.79	-0.95	1.42	15.62	-3.69
	Dog	*E. canis*	7.57	24.35	8.32	8.10	31.44	7.35
	Dog	*E. phagocytophila*	2.92	5.28	2.20	4.88	13.72	0.50
	Dog	*E. phagocytophila*	-2.09	-0.59	-2.47	-0.68	2.92	-2.84

Appendix 5.3 ELISA testing samples with antibodies against *E. phagocytophila* (Feral Goat) and *C. ruminantium* using peptides 89HGE and 89Cow

Sample	Ref.	Peptide				
		89 HGE	% reaction	89 Cow	% reaction	No peptide
E. phagocytophila positive sheep	935	0.28	9.30	0.23	4.09	0.18
	938	0.28	13.51	0.26	11.00	0.14
C. *ruminantium* positive goats	W	0.31	11.12	0.39	18.55	0.19
	G	0.33	9.53	0.38	14.83	0.22
E. phagocytophila positive dogs	m503	0.09	1.44	0.08	0.88	0.08
	m556	0.08	2.08	0.08	1.94	0.06
E. phagocytophila negative sheep	929	0.11	2.48	0.09	1.16	0.08
	938	0.13	2.41	0.12	1.56	0.11
C. ruminantium negative goats	W	0.22	5.28	0.16	-0.40	0.16
	G	0.10	-0.99	0.08	-3.03	0.11
E. phagocytophila negative dogs	24	0.15	4.38	0.14	3.76	0.10
	58	0.09	2.32	0.07	0.99	0.06

W *C. ruminantium,* Welgevonden strain
G *C. ruminantium*, Gardel strain

Appendix 5.4 Summary of serological results for Moredun Research Institute samples

Ref.	iELISA		cELISA			IFAT
	Mean OD 450 nm	>0.6	Mean OD 450nm	% inhibition	≥60 %inh.	
96/1137	0.62	+	0.21	80.20	+	+
96/1138	0.33	-	0.50	51.64	-	-
96/1139	0.43	-	0.87	15.90	-	-
96/1140	0.45	-	0.66	36.32	-	-
96/1141	0.49	-	0.45	56.98	-	-
96/1142	0.35	-	0.78	25.29	-	-
96/1143	0.52	-	0.47	54.87	-	-
96/1144	0.90	+	0.60	41.86	-	+
96/1145	0.30	-	0.63	39.79	-	-
96/1146	0.28	-	0.65	37.28	-	-
96/1147	0.36	-	0.49	52.31	-	-
96/1148	0.29	-	0.33	67.82	+	+
96/1149	0.58	-	0.48	53.37	-	-
96/1150	0.95	+	0.35	66.33	+	+
96/1151	0.66	+	0.94	9.73	-	-
96/1152	0.46	-	0.73	29.48	-	-
96/1153	0.41	-	0.42	59.73	-	+
96/1154	0.37	-	0.32	69.12	+	-
96/1155	0.41	-	1.06	-1.78	-	-
96/1156	0.53	-	0.86	17.39	-	-
96/1157	0.38	-	0.45	56.50	-	-
96/1158	0.27	-	0.34	67.49	+	+
96/1159	0.29	-	0.79	23.12	-	-
96/1160	0.51	-	0.74	28.61	-	-
96/1161	0.47	-	0.18	83.04	+	+
96/1162	0.45	-	0.85	18.26	-	-
96/1163	0.17	-	0.77	25.48	-	-
96/1164	0.61	+	0.09	91.67	+	+
96/1165	0.24	-	0.96	7.37	-	-
96/1166	0.26	-	0.84	18.93	-	-
96/1167	0.45	-	0.19	81.55	+	+
96/1168	0.33	-	0.69	34.01	-	-
96/1169	0.22	-	0.64	38.49	-	-
96/1170	0.14	-	0.69	33.24	-	+
96/1171	0.22	-	0.65	37.28	-	-
96/1172	0.25	-	0.68	34.68	-	-

Ref.	iELISA		cELISA			IFAT
	Mean OD 450 nm	>0.6	Mean OD 450nm	% inhibition	≥60 %inh.	
96/1173	0.13	-	0.58	44.46	-	+
96/1174	0.14	-	0.76	26.83	-	-
96/1175	0.24	-	0.54	47.83	-	-
96/1176	0.49	-	0.50	51.64	-	+
96/1843	0.29	-	0.09	91.92	+	+
96/1844	0.17	-	0.90	17.16	-	-
96/1845	0.25	-	1.13	-3.76	-	-
96/1846	0.15	-	0.77	28.91	-	-
96/1847	0.15	-	0.80	26.34	-	-
96/1848	0.22	-	0.91	16.75	-	
96/1849	0.34	-	0.76	30.24	-	
96/1850	0.37	-	0.90	17.35	-	
96/1851	0.92	+	0.41	61.96	+	-
96/1852	0.39	-	0.80	25.98	-	
96/1853	0.52	-	0.64	41.12	-	
96/1854	0.74	+	0.75	31.25	-	
96/1855	0.51	-	1.00	7.66	-	
96/1856	0.51	-	0.24	78.43	+	-
96/1857	0.22	-	0.83	24.09	-	
96/1858	0.39	-	0.61	43.92	-	
96/1859	0.57	-	0.66	39.33	-	
96/1860	0.89	+	0.26	75.95	+	+
96/1861	1.14	+	0.69	36.03	-	
96/1862	0.71	+	0.73	32.91	-	
96/224	0.40	-	0.43	42.50	-	
96/1444	0.29	-	0.67	9.73	-	
96/1445	0.76	+	0.57	20.74	-	
95/141	0.07	-	0.62	15.68	-	
95/142	0.06	-	0.83	-12.03	-	
96/478	0.50	-	0.19	73.31	+	+
96/1286	0.10	-	0.98	-33.45	-	
96/69	0.28	-	1.12	-50.95	-	
96/12	0.08	-	0.07	89.86	+	-
96/2	0.23	-	0.73	1.15	-	
96/25	0.55	-	0.72	2.97	-	
96/26	0.41	-	1.29	-73.65	-	

Ref.	iELISA		cELISA			IFAT
	Mean OD 450 nm	>0.6	Mean OD 450nm	% inhibition	≥60 %inh.	
96/27	0.33	-	0.83	-11.96	-	
96/28	0.21	-	1.16	-56.62	-	
96/29	0.91	+	1.06	-43.45	-	
96/30	0.34	-	1.24	-68.18	-	
96/31	0.51	-	0.92	-24.93	-	
96/32	0.65	+	0.39	46.15	-	
96/33	0.19	-	1.09	-46.96	-	
96/34	0.19	-	1.37	-84.53	-	
96/35	0.38	-	1.05	-41.35	-	
96/36	0.42	-	0.92	-23.99	-	
96/37	0.21	-	0.98	-32.16	-	
96/38	0.24	-	1.42	-91.69	-	
96/39	0.25	-	0.51	31.76	-	
buffalo	0.13	-	0.91	-23.31	-	
Arabian gazelle	0.11	-	1.15	-55.14	-	
Swamp deer	0.34	-	0.90	-22.03	-	
Sitatunga	0.14	-	0.79	-7.70	-	
Roan antelope	0.24	-	0.89	-20.61	-	
Roan antelope	0.26	-	0.93	-25.20	-	
Roan antelope	0.25	-	0.92	-24.12	-	
Roan antelope	0.14	-	0.88	-19.32	-	
Roan antelope	0.20	-	0.88	-18.38	-	
97/1345	0.29	-	0.28	74.02	+	-
97/1346	0.58	-	0.61	43.78	-	
97/1347	0.36	-	0.46	57.82	-	
97/1348	0.27	-	0.69	36.90	-	
97/1349	0.25	-	0.47	57.00	-	
97/1350	1.39	+	0.63	42.45	-	
97/1351	0.35	-	1.01	7.16	-	
97/1352	0.37	-	0.22	80.82	+	-
97/1353	0.66	+	0.12	88.71	+	+
97/1354	1.13	+	0.74	32.26	-	
97/1355	0.29	-	0.69	36.62	-	
97/1356	0.85	+	0.42	61.63	+	+
97/1357	0.24	-	0.55	49.56	-	
97/1358	0.64	+	0.57	47.41	-	
97/1359	0.85	+	0.39	64.16	+	-

Ref.	iELISA		cELISA			IFAT
	Mean OD 450 nm	>0.6	Mean OD 450nm	% inhibition	≥60 %inh.	
97/1360	0.54	-	0.59	45.85	-	
97/1361	0.39	-	0.88	19.55	-	
97/1362	0.77	+	0.50	54.11	-	
97/1363	0.57	-	0.45	58.51	-	
97/1364	0.81	+	0.63	41.95	-	
97/1365	0.49	-	0.84	19.12	-	
97/1366	0.16	-	0.78	24.90	-	
97/1367	0.23	-	0.82	20.81	-	
97/1368	0.19	-	0.36	66.87	+	-
97/1369	0.51	-	0.81	25.33	-	
97/1370	0.23	-	0.73	33.36	-	
97/1371	0.31	-	0.27	63.51	+	+
97/1372	0.37	-	0.86	-16.15	-	
97/1373	0.35	-	0.91	-22.91	-	
97/1374	0.22	-	0.57	22.70	-	
97/1375	0.38	-	0.48	34.53	-	
97/1376	0.43	-	0.53	28.99	-	
97/1377	0.26	-	0.54	28.50	-	
97/1378	1.07	+	0.26	65.73	+	+
97/1379	0.80	+	0.28	62.97	+	+
97/1380	0.40	-	0.72	5.25	-	
97/1381	0.33	-	0.76	-0.13	-	
97/1382	0.18	-	0.81	-6.37	-	
97/1383	0.41	-	0.66	13.72	-	
97/1384	0.20	-	0.93	-22.59	-	
11 1980	0.28	-	0.66	13.20	-	
470 1988	0.13	-	0.87	-13.79	-	
7220 1979	0.15	-	0.96	-26.20	-	
97/50	0.25	-	1.02	-33.95	-	
97/310	1.63	+	0.22	71.70	+	+
97/311	0.67	+	0.50	34.27	-	
97/199 Gnu	0.12	-	0.51	32.76	-	
97/200 Gnu	0.15	-	0.49	36.18	-	
97/117	0.07	-	0.62	18.32	-	
Roan antelope	0.24	-	0.91	-19.57	-	
89/148	0.22	-	0.55	27.97	-	

Appendix 5.5 c-ELISA preliminary results using experimental sheep and roe deer samples (Test 1)

Sheep controls	OD 450 nm	% inhibition	Roe deer	OD 450 nm	% inhibition	Cut-off >50%
931(pre)	1.28	0.00	1	1.12	12.55	-
928 (1)[1]	0.88	31.76	2	0.99	23.15	-
929 (1)	1.06	17.15	3	1.09	15.24	-
931 (1)	1.05	18.51	4	1.27	0.78	-
933 (1)	1.21	5.92	5	0.79	38.39	-
935 (1)	0.95	25.88	6	0.39	68.90	+
937 (1)	1.14	11.22	7	0.17	87.06	+
938 (1)	0.91	28.80	8	1.09	15.08	-
930 (3)[2]	0.13	89.71	9	0.33	74.16	+
937 (3)	0.28	78.53	10	1.20	6.35	-
938 (3)	0.35	72.45	11	1.24	3.23	-
936 (4)[3]	0.26	79.42	12	1.01	21.01	-
937 (4)	0.33	74.01	13	1.22	4.79	-
938 (4)	0.33	74.24	14	1.17	8.96	-
939 (4)	0.27	79.35	15	0.22	82.85	+
937 (21)[4]	0.28	78.57	16	0.29	77.63	+
			17	1.22	4.91	-
			18	0.96	25.60	-
			19	0.99	23.11	-
			20	0.44	65.71	+
			21	0.25	80.51	+
			22	0.85	34.14	-
			23	0.25	80.63	+
			24	0.53	58.42	+

[1] 1 week after experimental inoculation
[2] 3 weeks after experimental inoculation
[3] 4 weeks after experimental inoculation
[4] 21 weeks after experimental inoculation

Appendix 5.6 Summary of serological results for wild roe deer samples (Test 2)

	cELISA		Cut-off	iELISA	Cut-off	
Sample No.	OD 450 nm	% inhibition	>50%	OD 450 nm	≥0.45	IFAT
1	1.12	12.55	-	0.09	-	-
2	0.99	23.15	-	0.20	-	+
3	1.09	15.24	-	0.12	-	-
4	1.27	0.78	-	0.08	-	-
5	0.79	38.39	-	0.09	-	-
6	0.34	68.90	+	0.24	-	+
7	0.17	87.06	+	1.25	+	+
8	0.43	40.46	-	0.09	-	-
9	0.29	60.03	+	0.23	-	+
10	0.79	-10.13	-	0.10	-	-
11	0.82	-13.67	-	0.14	-	-
12	0.67	6.59	-	0.07	-	-
13	0.79	-9.44	-	0.11	-	-
14	0.79	-9.51	-	0.13	-	-
15	0.21	70.71	+	0.14	-	+
16	0.34	52.87	+	0.41	+	+
17	0.73	-1.80	-	0.05	-	-
18	1.09	-50.87	-	0.05	-	-
19	0.41	42.69	-	0.11	-	-
20	1.24	-71.41	-	0.09	-	-
21	0.91	-25.95	-	0.27	-	-
22	0.15	79.88	+	0.10	-	-
23	0.19	73.98	+	0.21	-	-
24	0.21	70.92	+	0.39	-	-
25	0.27	61.97	+	0.64	+	+
26	0.23	67.59	+	0.53	+	+
27	0.26	64.54	+	0.72	+	+
28	0.19	74.05	+	0.46	+	+
29	0.11	84.59	+	0.98	+	+
30	0.14	80.29	+	0.60	+	+
31	0.17	76.68	+	1.02	+	+
32	0.08	89.31	+	0.90	+	+
33	0.50	30.53	-	0.57	+	+
34	0.19	73.28	+	1.03	+	+
35	0.18	74.81	+	1.00	+	+
36	0.35	51.56	+	0.49	+	+
37	0.11	84.80	+	0.87	+	+
38	0.08	88.41	+	0.37	-	-
39	0.08	88.97	+	0.33	-	+
40	0.10	86.05	+	1.52	+	+
41	0.17	76.82	+	0.78	+	-
42	0.89	-23.31	-	0.28	-	+
43	0.10	85.64	+	0.86	+	+

	cELISA		Cut-off	iELISA	Cut-off	
Sample No.	OD 450 nm	% inhibition	>50%	OD 450 nm	≥0.45	IFAT
44	0.24	66.62	+	0.93	+	-
45	0.08	88.48	+	0.75	+	+
46	0.11	84.25	+	0.60	+	-
48	0.16	77.38	+	1.23	+	+
49	0.11	84.59	+	1.56	+	+
51	0.29	59.13	+	1.69	+	+
52	0.32	55.31	+	0.79	+	+
53	0.11	85.01	+	1.50	+	+
54	0.13	82.03	+	0.42	-	-
55				0.73	+	-
56				1.04	+	+
57				1.76	+	+
58				0.14	-	-
59				0.45	-	-
60				0.21	-	-
61				0.14	-	-
62				0.13	-	-
63				0.39	-	-
64				0.27	-	-
65				0.75	+	+
66				0.76	+	+
67				0.31	-	-
68				1.44	+	+
69				1.76	+	+
70				0.46	+	+
71				0.61	+	+
72				0.43	-	-
73				0.72	+	+
74				0.72	+	-
75				1.26	+	+
76				0.72	+	+
77				0.86	+	+
78				0.81	+	+
79				0.95	+	+
80				0.19	-	+
81				0.39	-	+
82				0.76	+	-
83				0.82	+	-
84				0.74	+	-
85				0.83	+	+
86				1.19	+	+
87				1.21	+	-
88				0.72	+	+

Appendix 5.7 cELISA with MAb R5A9 and feline samples

Cat No.	OD 450 nm	% inhibition	Cut-off ≥60%	IFAT	FIV
F9570E	0.79	26.03	-	-	+
F4098F	0.64	39.63	-	-	-
F3918F	0.37	64.87	+	-	-
F3880F	0.53	50.00	-	-	-
F3961F	0.57	46.44	-	-	-
M670	0.82	23.55	-	-	
F3909F	0.66	38.13	-	-	-
F3935F	0.36	66.09	+	+	-
F4136F	1.10	-3.52	-	-	-
F9408E	0.98	8.35	-	+	+
M418	1.06	0.33	-	-	
F3911F	0.82	22.89	-	-	-
F3981F	0.82	23.31	-	-	-
F9321E	0.53	50.75	-	-	+
F3869F	0.53	50.05	-	-	-
F3881F	0.33	68.71	+	+	-
F3875F	0.39	62.57	+	-	-
F3917F	0.64	39.96	-	-	-
F4096F	1.06	0.75	-	-	-
F4140F	0.78	26.55	-	-	-
F3780F	0.52	51.03	-	-	-
F9446E	0.61	42.78	-	-	+
F3938F	0.64	40.19	-	-	-
53	1.42	-33.54	-	-	
F4142F	0.46	56.61	-	-	-
F9443E	0.60	43.39	-	-	+
F3816F	0.33	69.47	+	+	-
F4139F	0.28	73.45	+	-	-
M546	0.67	36.87	-	-	
F9331E	0.29	79.33	+	+	+
F3889F	0.41	70.46	+	-	-
F9547E	0.73	47.28	-	-	+
F3913F	0.44	68.46	+	+	-
F9360E	0.88	36.06	-	-	+
M443	0.99	27.84	-	-	
F4056F	0.52	62.67	+	-	-
F9493E	0.65	53.08	-	-	+

Cat No.	OD 450 nm	% inhibition	Cut-off ≥60%	IFAT	FIV
F3887F	0.25	81.75	+	-	-
F9333E	0.41	70.02	+	+	+
M392	0.59	56.77	-	-	
F4566E	0.85	38.34	-	-	+
F9317E	0.86	37.73	-	-	+
F4090F	1.34	2.79	-	-	-
F9545E	0.75	45.87	-	-	+
F9505E	0.41	70.60	+	-	+
F4009F	2.31	-67.27	-	-	-
F3863F	0.41	69.99	+	+	-
F3840F	0.38	72.81	+	+	-
F3842F	0.71	48.69	-	-	
F4077F	0.27	80.77	+	+	-
F4054F	0.24	82.87	+	+	-
F9341E	0.89	35.08	-	-	+
F3687F	0.34	75.42	+	-	-
F3838F	0.27	80.49	+	-	-
F4011F	0.33	76.43	+	+	-
F9381E	1.07	22.19	-	-	+
M397	0.60	56.23	-	-	

Appendix 5.8 cELISA using MAb R5A9 and canine samples

Dog No.	OD 450 nm	% inhibition	Dog No.	OD 450 nm	% inhibition
19	0.83	1.02	M229	0.09	86.52
92	1.06	-26.45	M291	0.95	-33.73
105	1.11	-33.27	M352	0.87	-22.72
111	1.06	-26.57	M434	0.78	-9.88
118	0.97	-15.56	M526	0.55	22.16
129	0.07	91.38	M695	0.61	13.83
142	0.80	4.249	M29	0.91	-28.02
161	0.85	-2.095	M386	1.11	-57.02
198	0.73	13.17	M20	0.69	3.39
M503	0.83	0.658	M228	0.74	-4.02
M287/3	0.22	73.25	M664	0.58	17.71
M428	0.14	83.66	M561	0.96	-35.85
M445	0.91	-8.26	M865	0.54	23.57
M1865	0.46	44.46	M704	1.10	-54.98
M525	0.96	-14.48	M686	0.59	15.74
M556	0.81	2.99	M962	0.36	48.69
M125	0.83	1.08	140	0.55	22.94
169	0.55	33.81	136	0.40	43.54
187	0.41	51.41	146	0.63	10.80
171	0.58	30.46	148	0.56	20.75
152	0.67	20.35	143	0.74	-4.23
144	0.37	55.89	138	0.63	11.50
141	0.84	0.00	132	0.58	17.85
135	0.49	41.41	128	0.86	-21.88
168	0.40	52.12	134	0.66	7.06
200	0.80	3.89	149	0.59	16.09
153	0.55	33.93	137	0.59	16.87
167	0.61	27.29	133	0.47	33.31
139	0.37	56.13	125	0.55	21.81
176	0.63	24.36	145	0.56	21.31
185	0.53	36.62	131	0.61	14.47
165	0.47	43.39	150	0.63	11.64
159	0.60	27.71	162	0.59	17.08
191	0.21	75.46	174	0.50	29.43
172	0.42	49.55	166	0.50	29.22
182	0.65	22.68	155	0.68	3.53
156	0.48	42.19	157	0.59	17.15
178	0.64	23.22	154	0.66	7.27
164	0.80	3.83	173	0.57	20.11
124	0.49	40.63	177	0.62	12.28
147	0.57	31.42	170	0.51	27.52
130	0.57	32.14	172	0.52	26.25
126	0.62	25.73	175	0.63	11.50
160	0.55	34.65	163	0.48	32.53
151	0.64	23.82	158	0.53	25.55

Appendix 7.1 Sequence alignment of a 773-bp fragment after removal of primers (410-bp for the canine sample from Utrecht) of *groEL* operon gene using Lasergene DNAstar software package (Clustal method)

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1
AACATTGTCTGCAAATGGAGACAAGAACATAGGAAGTAAGATTGCACAGT      Ehr/8
AACATTGTCTGCAAATGGAGACAAGAACATAGGAAGTAAGATTGCACAGT      FG1
AACATTGTCTGCAAATGGAGACAAGAACATAGGAAGTAAGATTGCACAGC      FG2
AACATTGTCTGCAAATGGAGACAAGAACATAGGAAGTAAGATTGCACAGT      OS
AACATTATCTGCAAATGGAGACAAGAACATAGGAAGTAAGATTGCACAGT      Harris
AACATTGTCTGCAAATGGAGACAAGAACATAGGAAGTAAGATTGCACAGT      C8
AACATTGTCTGCAAATGGAGACAAGAACATAGGAAGTAAGATTGCACAGT      Perth
AACATTGTCTGCGAATGGAGACAAGAACATAGGAAGTAAGATTGCACAGT      HGE[a]
AACATTGTCTGCGAATGGAGACAAGAACATAGGAAGTAAGATTGCACAGT      HGE[b]
AACATTGTCTGCGAATGGAGACAAGAACATAGGAAGTAAGATTGCACAGT      HGE[c]
AACATTGTCTGCGAATGGAGACAAGAACATAGGAAGTAAGATTGCACAGT      SH
AACATTGTCTGCGAATGGAGACAAGAACATAGGAAGTAAGATTGCACAGT      SwH
--------------------------------------------------      Canine
AACATTGTCTGCGAATGGAGACAAGAACATAGGAAGTAAGATTGCACAGT      E. equi
AACTATCTCTGCGAATGGTGATAAAAATATAGGTACTAAAATTGCACAGT      C. rum

51
GTGTTAAAGAAGTCGGTAAAGACGGTGTTATAACTGTTGAAGAAAGCAAA      Ehr/8
GTGTTAAAGAAGTCGGTAAAGACGGTGTTATAACTGTTGAAGAAAGCAAA      FG1
GTGTTAAAGAAGTCGGTAAAGACGGTGTTATAACTGTTGAAGAAAGCAAA      FG2
GTGTTAAAGAAGTCGGTAAAGACGGTGTTATAACTGTTGAAGAAAGCAAA      OS
GTGTTAAAGAAGTCGGTAAAGACGGTGTTATAACTGTTGAAGAAAGCAAA      Harris
GTGTTAAAGAAGTCGGTAAAGACGGTGTTATAACTGTTGAAGAAAGCAAA      C8
GTGTTAAAGAAGTCGGTAAAGACGGTGTTATAACTGTTGAAGAAAGCAAA      Perth
GTGTTAAAGAAGTCGGTAAAGACGGTGTTATAACTGTTGAAGAAAGCAAA      HGE[a]
GTGTTAAAGAAGTCGGTAAAGACGGTGTTATAACTGTTGAAGAAAGCAAA      HGE[b]
GTGTTAAAGAAGTCGGTAAAGACGGTGTTATAACTGTTGAAGAAAGCAAA      HGE[c]
GTGTTAAAGAAGTCGGTAAAGACGGTGTTATAACTGTTGAAGAAAGCAAA      SH
GTGTTAAAGAAGTCGGTAAAGACGGTGTTATAACTGTTGAAGAAAGCAAA      SwH
--------------------------------------------------      Canine
GTGTTAAAGAAGTCGGTAAAGACGGTGTTATAACTGTTGAAGAAAGCAAA      E. equi
GTGTTAAAGAAGTTGGTAAAGATGGGGTTATTACAGTTGAAGAAAGCAAA      C. rum
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101
GGCTTCAAGGATCTAGAAGTTGAAAAGACTGATGGTATGCAGTTTGATCG      Ehr/8
GGCTTCAAGGATCTAGAAGTTGAAAAGACTGATGGTATGCAGTTTGATCG      FG1
GGCTTCAAGGATCTAGAAGTTGAAAAGACTGATGGTATGCAGTTTGATCG      FG2
GGCTTCAAGGATCTAGAAGTTGAAAAGACTGATGGTATGCAGTTTGATCG      OS
GGCTTCAAGGATCTAGAAGTTGAAAAGACTGATGGTATGCAGTTTGATCG      Harris
GGCTTCAAGGATCTAGAAGTTGAAAAGACTGATGGTATGCAGTTTGATCG      C8
GGCTTCAAGGATCTAGAAGTTGAAAAGACTGATGGTATGCAGTTTGATCG      Perth
GGCTTCAAGGATCTAGAAGTTGAAAAGACTGATGGTATGCAGTTTGATCG      HGE[a]
GGCTTCAAGGATCTAGAAGTTGAAAAGACTGATGGTATGCAGTTTGATCG      HGE[b]
GGCTTCAAGGATCTAGAAGTTGAAAAGACTGATGGTATGCAGTTTGATCG      HGE[c]
GGCTTCAAGGATCTAGAAGTTGAAAAGACTGATGGTATGCAGTTTGATCG      SH
GGCTTCAAGGATCTAGAAGTTGAAAAGACTGATGGTATGCAGTTTGATCG      SwH
--------------------------------------------------      Canine
GGCTTCAAGGATCTAGAAGTTGAAAAGACTGATGGTATGCAGTTTGATCG      E. equi
GGATTTAAAGAGTTAGATGTTGAAAAGACTGATGGTATGCAATTTGATAG      C. rum
151
CGGATATCTTTCGCCTTACTTTGTTACAAATGCTGAAAAAATGCTGGTGG      Ehr/8
CGGATATCTTTCGCCTTACTTTGTTACAAATGCTGAAAAAATGCTGGTGG      FG1
CGGATATCTTTCGCCTTACTTTGTTACAAATGCTGAAAAAATGCTGGTGG      FG2
CGGATATCTTTCGCCTTACTTTGTTACAAATGCTGAAAAAATGCTGGTGG      OS
CGGATATCTTTCGCCTTACTTTGTTACAAATGCTGAAAAAATGCTGGTGG      Harris
CGGATATCTTTCGCCTTACTTTGTTGCAAATGCTGAAAAAATGCTGGTGG      C8
CGGATATCTTTCGCCTTACTTTGTTACAAATGCTGAAAAAATGCTGGTGG      Perth
CGGATATCTTTCGCCTTACTTTGTTACAAATGCTGAAAAAATGCTGGTGG      HGE[a]
CGGATATCTTTCGCCTTACTTTGTTACAAATGCTGAAAAAATGCTGGTGG      HGE[b]
CGGATATCTTTCGCCTTACTTTGTTACAAATGCTGAAAAAATGCTGGTGG      HGE[c]
CGGATATCTTTCGCCTTACTTTGTTACAAATGCTGAAAAAATGCTGGTGG      SH
CGGATATCTTTCGCCTTACTTTGTTACAAATGCTGAAAAAATGCTGGTGG      SwH
--------------------------------------------------      Canine
CGGATATCTTTCGCCTTACTTTGTTACAAATGCTGAAAAAATGCTGGTGG      E. equi
AGGATATCTTTCTCCTTATTTTGTTACTAACTCAGAAAAGATGTTAGTTG      C. rum
201
AATTTGAAAATCCATACATATTCCTTACTGAAAAGAAGATTAATCTTGTA      Ehr/8
AATTTGAAAATCCATACATATTCCTTACTGAAAAGAAGATTAATCTTGTA      FG1
AATTTGAAAATCCATACATATTCCTTACTGAAAAGAAGATTAATCTTGTA      FG2
AATTTGAAAATCCATACATATTCCTTACTGAAAAGAAGATTAATCTTGTA      OS
AATTTGAAAATCCATACATATTCCTTACTGAAAAGAAGATTAATCTTGTA      Harris
AATTTGAAAATCCATACATATTCCTTACTGAAAAGAAGATTAATCTTGTA      C8
AATTTGAAAATCCATACATATTCCTTACTGAAAAGAAGATTAATCTTGTA      Perth
AATTTGAAAATCCATACATATTCCTTACTGAAAAGAAGATTAATCTTGTA      HGE[a]
AATTTGAAAATCCATACATATTCCTTACTGAAAAGAAGATTAATCTTGTA      HGE[b]
AATTTGAAAATCCATACATATTTCTTACTGAAAAGAAGATTAATCTTGTA      HGE[c]
AATTTGAAAATCCATACATATTTCTTACTGAAAAGAAGATTAATCTTGTA      SH
AATTTGAAAATCCATACATATTTCTTACTGAAAAGAAGATTAATCTTGTA      SwH
--------------------------------------------------      Canine
AATTTGAAAATCCATACATATTCCTTACTGAAAAGAAGATTAATCTTGTA      E. equi
AGTTTGAAAATCCTTATATTTTATTAACAGAAAAGAAACTAAATATAATA      C. rum
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251
CAAAGCATTCTACCAATCTTAGAAAACGTTGCACGGTCTGGAAGACCATT   Ehr/8
CAAAGCATTCTACCAATCTTAGAAAACGTTGCACGGTCTGGAAGACCATT   FG1
CAAAGCATTCTACCAATCTTAGAAAACGTTGCACGGTCTGGAAGGCCATT   FG2
CAAAGCATTCTACCAATCTTAGAAAACGTTGCACGGTCTGGAAGGCCATT   OS
CAAAGCATTCTACCAATCTTAGAAAACGTTGCACGGTCTGGAAGGCCATT   Harris
CAAAGCATTCTACCAATCTTAGAAAACGTTGCACGGTCTGGAAGACCATT   C8
CAAAGCATTCTACCAATCTTAGAAAACGTTGCACGGTCTGGAAGGCCATT   Perth
CAAAGCATTCTACCAATCTTAGAAAACGTTGCACGGTCTGGAAGACCATT   HGE[a]
CAAAGCATTCTACCAATCTTAGAAAACGTTGCACGGTCTGGAAGACCATT   HGE[b]
CAAAGCATTCTACCAATCTTAGAAAACGTTGCACGGTCTGGAAGACCATT   HGE[c]
CAAAGCATTCTACCAATCTTAGAAAACGTTGCACGGTCTGGAAGACCATT   SH
CAAAGCATTCTACCAATCTTAGAAAACGTTGCACGGTCTGGAAGACCATT   SwH
----------------------------------------------------   Canine
CAAAGCATTCTACCAATCTTAGAAAACGTTGCACGGTCTGGAAGACCATT   E. equi
CAACCTCTATTGCCAATTTTAGAGAATATTGCTAGATCAGGTAGACCACT   C. rum

301
GCTCATCATAGCTGAAGACGTTGAAGGTGAAGCTCTGAGCACGCTTGTAC   Ehr/8
GCTCATCATAGCTGAAGACGTTGAAGGTGAAGCTCTGAGCACGCTTGTAC   FG1
GCTCATCATAGCTGAAGACGTTGAAGGTGAAGCTCTGAGCACGCTTGTAC   FG2
GCTCATCATAGCTGAAGACGTTGAAGGTGAAGCTCTTAGCACGCTTGTAC   OS
GCTCATCATAGCTGAAGACGTTGAAGGTGAAGCTCTTAGCACGCTTGTAC   Harris
GCTCATCATAGCTGAAGACGTTGAAGGTGAAGCTCTGAGCACGCTTGTAC   C8
GCTCATCATAGCTGAAGACGTTGAAGGTGAAGCTCTTGGCACGCTTGTAC   Perth
GCTCATCATAGCTGAAGACGTTGAAGGTGAAGCTCTGAGCACGCTTGTAC   HGE[a]
GCTCATCATAGCTGAAGACGTTGAAGGTGAAGCTCTGAGCACGCTTGTAC   HGE[b]
GCTCATCATAGCTGAAGACGTTGAAGGTGAAGCTCTGAGCACGCTTGTAC   HGE[c]
GCTCATCATAGCTGAAGACGTTGAAGGTGAAGCTCTGAGCACGCTTGTAC   SH
GCTCATCATAGCTGAAGACGTTGAAGGTGAAGCTCTGAGCACGCTTGTAC   SwH
----------------------------------------**TGTAC**   Canine
GCTCATCATAGCTGAAGACGTTGAAGGTGAAGCTCTGAGCACGCTTGTAC   E. equi
TTTAATCATTGCAGAGGATGTAGAAGGTGAAGCACTTAGTACACTTGTAT   C. rum

351
TCAATAAGCTCCGTGGTGGCCTTCAAGTTGCTGCTGTAAAGGCGCCTGGT   Ehr/8
TCAATAAGCTCCGTGGTGGCCTTCAAGTTGCTGCTGTAAAGGCGCCTGGT   FG1
TCAATAAGCTCCGTGGTGGCCTTCAAGTTGCTGCTGTAAAGGCGCCTGGT   FG2
TCAATAAGCTCCGTGGTGGCCTTCAAGTTGCTGCTGTAAAGGCGCCTGGT   OS
TCAATAAGCTCCGTGGTGGCCTTCAAGTTGCTGCTGTAAAGGCGCCTGGT   Harris
TCAATAAGCTCCGTGGTGGCCTTCAAGTTGCTGCTGTAAAGGCGCCTGGT   C8
TCAATAAGCTCCGTGGTGGCCTTCAAGTTGCTGCTGTAAAGGCGCCTGGT   Perth
TCAATAAGCTCCGTGGTGGCCTTCAAGTTGCTGCTGTAAAGGCGCCTGGT   HGE[a]
TCAATAAGCTCCGTGGTGGCCTTCAAGTTGCTGCTGTAAAGGCGCCTGGT   HGE[b]
TCAATAAGCTCCGTGGTGGCCTTCAAGTTGCTGCTGTAAAGGCGCCTGGT   HGE[c]
TCAATAAGCTCCGTGGTGGCCTTCAAGTTGCTGCTGTAAAGGCGCCTGGT   SH
TCAATAAGCTCCGTGGTGGCCTTCAAGTTGCTGCTGTAAAGGCGCCTGGT   SwH
**TCAATAAGCTCCGTGGTG**GCCTTCAAGTTGCTGCTGTAAAGGCGCCTGGT   Canine
TCAATAAGCTCCGTGGTGGCCTTCAAGTTGCTGCTGTAAAGGCGCCTGGT   E. equi
TGAATAAGTTGCGTGGTGGTTTACATGTAGCTGCAGTTAAAGCTCCAGGG   C. rum
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401

TTCGGTGACAGGAGAAAAGACATGCTTGGCGATATTGCTGTAATAGTAGG	Ehr/8
TTTGGTGACAGGAGAAAAGACATGCTTGGCGATATTGCTGTAATAGTAGG	FG1
TTCGGTGACAGGAGAAAAGACATGCTTGGCGATATTGCTGTAATAGTAGG	FG2
TTTGGTGACAGGAGAAAAGACATGCTTGGCGATATTGCTGTAATAGTAGG	OS
TTTGGTGACAGGAGAAAAGACATGCTTGGCGATATTGCTGTAATAGTAGG	Harris
TTTGGTGACAGGAGAAAAGACATGCTTGGCGATATTGCTGTAATAGTAGG	C8
TTTGGTGACAGGAGAAAAGACATGCTTGGCGATATTGCTGTAATAGTAGG	Perth
TTCGGTGACAGAAGAAAAGACATGCTTGGCGATATTGCTGTAATAGTAGG	HGE[a]
TTCGGTGACAGAAGAAAAGACATGCTTGGCGATATTGCTGTAATAGTAGG	HGE[b]
TTCGGTGACAGGAGAAAAGACATGCTTGGCGATATTGCTGTAATAGTAGG	HGE[c]
TTCGGTGACAGGAGAAAAGACATGCTTGGCGATATTGCTGTAATAGTAGG	SH
TTCGGTGACAGGAGAAAAGACATGCTTGGCGATATTGCTGTAATAGTAGG	SwH
TTCGGTGACAGGAGAAAAGACATGCTTGGCGATATTGCTGTAATAGTAGG	Canine
TTCGGTGACAGAAGAAAAGACATGCTTGGCGATATTGCTGTAATAGTAGG	*E. equi*
TTTGGAGATAGAAGAAAAGATATGTTAGGTGATATTGCAATTTTAACTGG	*C. rum*

451

CGCTAAGTATGTAGTAAATGACGAGCTTGCTGTTAAGATGGAAGACATCG	Ehr/8
CGCTAAGTATGTAGTAAATGACGAGCTTGCTGTTAAGATGGAAGATATCG	FG1
CGCTAAGTATGTAGTAAATGACGAGCTTGCTGTTAAGATGGAAGACATCG	FG2
CGCTAAGTATGTAGTAAATGACGAGCTTGCTGTTAAGATGGAAGACATCG	OS
CGCTAAGTATGTAGTAAATGACGAGCTTGCTGTTAAGATGGAAGACATCG	Harris
CACTAAGTATGTAGTAAATGACGAGCTTGCTGTTAAGATGGAAGATATCG	C8
CGCTAAGTATGTAGTAAATGACGAGCTTGCTGTTAAGATGGAAGATATCG	Perth
CGCTAAGTATGTAGTAAATGACGAGCTTGCTGTTAAGATGGAAGACATCG	HGE[a]
CGCTAAGTATGTAGTAAATGACGAGCTTGCTGTTAAGATGGAAGACATCG	HGE[b]
CGCTAAGTATGTAGTAAATGACGAGCTTGCTGTTAAGATGGAAGACATCG	HGE[c]
CGCTAAGTATGTAGTAAATGACGAGCTTGCTGTTAAGATGGAAGACATCG	SH
CGCTAAGTATGTAGTAAATGACGAGCTTGCTGTTAAGATGGAAGACATCG	SwH
CGCTAAGTATGTAGTAAATGACGAGCTTGCTGTTAAGATGGAAGACATCG	Canine
CGCTAAGTATGTAGTAAATGACGAGCTTGCTGTTAAGATGGAAGACATCG	*E. equi*
AGCTAAACATGTAATAAATGATGAGCTTGCTATTAAAATGGAAGATTTGA	*C. rum*

501

CTCTAAGCGATCTTGGTACTGCTAAGAGCGTACGCATCACAAAAGACGCA	Ehr/8
CTCTAAGCGATCTTGGTACTGCTAAGAGCGTACGCATCACAAAAGACGCA	FG1
CTCTAAGCGATCTTGGTACTGCTAAGAGCGTACGCATCACAAAAGACGCA	FG2
CTCTAAGCGATCTTGGTACTGCTAAGAGCGTACGCATCACAAAAGACGCA	OS
CTCTAAGCGATCTTGGTACTGCTAAGAGCGTACGCATCACAAAAGACGCA	Harris
CTCTAAGCGATCTTGGTACTGCTAAGAGCGTACGCATCACAAAAGACGCA	C8
CTCTAAGCGATCTTGGTACTGCTAAGAGCGTACGCATCACAAAAGACGCA	Perth
CTCTAAGCGATCTTGGTACTGCTAAGAGCGTGCGAATCACAAAAGACGCA	HGE[a]
CTCTAAGCGATCTTGGTACTGCTAAGAGCGTGCGAATCACAAAAGACGCA	HGE[b]
CTCTAAGCGATCTTGGTACTGCTAAGAGCGTACGCATCACAAAAGACGCA	HGE[c]
CTCTAAGCGATCTTGGTACTGCTAAGAGCGTACGCATCACAAAAGACGCA	SH
CTCTAAGCGATCTTGGTACTGCTAAGAGCGTACGCATCACAAAAGACGCA	SwH
CTCTAAGCGATCTTGGTACTGCTAAGAGCGTACGCATCACAAAAGACACA	Canine
CTCTAAGCGATCTTGGTACTGCTAAGAGCGTGCGAATCACAAAAGACGCA	*E. equi*
CTCTATGTGATTTAGGAACTGCTAAGAATATACGTATTACAAAAGATACT	*C. rum*

551

ACTACTATCATAGGCAGTGTTGATAGCAGTTCTGAAAGCATAGCTAGCAG	Ehr/8
ACTACTATCATAGGTAGTGTTGATAGCAGTTCTGAAAGCATAGCTAGCAG	FG1
ACTACTATCATAGGCAGTGTTGATAGCAGTTCTGAAAGCATAGCTAGCAG	FG2
ACTACTATCATAGGCAGTGTTGATAGCAGTTCTGAAAGCATAGCTAGCAG	OS
ACTACTATCATAGGCAGTGTTGATAGCAGTTCTGAAAGCATAGCTAGCAG	Harris
ACTACTATCATAGGTAGTGTTGATAGCAGTTCTGAAAGCATAGCTAGCAG	C8
ACTACTATCATAGGTAGTGTTGATAGCAGTTCTGAAAGCATAGCTAGCAG	Perth
ACTACTATTATAGGTAGTGTTGATAGCAGTTCTGAAAGCATAGCTAGCAG	HGE[a]
ACTACTATTATAGGTAGTGTTGATAGCAGTTCTGAAAGCATAGCTAGCAG	HGE[b]
ACTACTATCATAGGTAGTGTTGATAGCAGTTCTGAAAGCATAGCTAGCAG	HGE[c]
ACTACTATCATAGGTAGTGTTGATAGCAGTTCTGAAAGCATAGCTAGCAG	SH
ACTACTATCATAGGTAGTGTTGATAGCAGTTCTGAAAGCATAGCTAGCAG	SwH
ACTACTATCATAGGTAGTGTTGATAGCAGTTCTGAAAGCATAGCTAGCAG	Canine
ACTACTATTATAGGTAGTGTTGATAGCAGTTCTGAAAGCATAGCTAGCAG	*E. equi*
ACAACTATTATTGGTAGTGTAGATAATAGTTGTGCTCATGTTCAAAGTAG	*C. rum*

601

GACTAATCAAATCAAAGCTCAGATAGAAAATTCTAGTTCTGATTATGACA	Ehr/8
GACTAATCAAATCAAAGCTCAGATAGAAAATTCTAGTTCTGATTATGACA	FG1
GACTAATCAAATCAAAGCTCAGATAGAAAATTCTAGTTCTGATTATGACA	FG2
GACTAATCAAATCAAAGCTCAGATAGAAAATTCTAGTTCTGATTATGACA	OS
GACTAATCAAATCAAAGCTCAGATAGAAAATTCTAGTTCTGATTATGACA	Harris
GACTAATCAAATCAAAGCTCAGATAGAAAATTCTAGTTCTGATTATGACA	C8
GACTAATCAAATCAAAGCTCAGATAGAAAATTCTAGTTCTGATTATGACA	Perth
GACTAATCAAATCAAAGCTCAGATAGAAAATTCTAGTTCTGATTATGACA	HGE[a]
GACTAATCAAATCAAAGCTCAGATAGAAAATTCTAGTTCTGATTATGACA	HGE[b]
GACTAATCAAATCAAAGCTCAGATAGAAAATTCTAGTTCTGATTATGACA	HGE[c]
GACTAATCAAATCAAAGCTCAGATAGAAAATTCTAGTTCCGATTATGACA	SH
GACTAATCAAATCAAAGCTCAGATAGAAAATTCTAGTTCTGATTATGACA	SwH
GACTAATCAAATCAAAGCTCAGATAGAAAATTCTAGTTCTGATTATGACA	Canine
GACTAATCAAATCAAAGCTCAGATAGAAAATTCTAGTTCTGATTATGACA	*E. equi*
AATTTGTCAAATTAGAATGCAAATTGATAATTCTACTTCAGATTATGATA	*C. rum*

651

AGGAAAAGCTTAGAGAACGTTTAGCAAAGCTTTCCGGTGGTGTTGCTGTA	Ehr/8
AGGAAAAGCTTAGAGAACGTTTAGCGAAGCTTTCCGGTGGCGTTGCTGTA	FG1
AGGAAAAGCTTAGAGAACGTTTAGCGAAGCTTTCCGGTGGCGTTGCTGTA	FG2
AGGAAAAGCTTAGAGAACGTTTAGCAAAGCTTTCCGGTGGTGTTGCTGTA	OS
AGGAAAAGCTTAGAGAACGTTTAGCAAAGCTTTCCGGTGGTGTTGCTGTA	Harris
AGGAAAAGCTTAGAGAACGTTTAGCGAAGCTTTCCGGTGGCGTTGCTGTA	C8
AGGAAAAGCTTAGAGAACGTTTAGCGAAGCTTTCTGGTGGCGTTGCTGTA	Perth
AGGAAAAGCTTAGAGAACGTTTAGCGAAGCTTTCCGGTGGCGTTGCTGTA	HGE[a]
AGGAAAAGCTTAGAGAACGTTTAGCGAAGCTTTCCGGTGGCGTTGCTGTA	HGE[b]
AGGAAAAGCTTAGAGAACGTTTAGCAAAGCTTTCCGGTGGTGTTGCTGTA	HGE[c]
AGGAAAAGCTTAGAGAACGTTTAGCAAAGCTTTCCGGTGGTGTTGCTGTA	SH
AGGAAAAGCTTAGAGAACGTTTAGCAAAGCTTTCCGGTGGTGTTGCTGTA	SwH
AGGAAAAGCTTAGAGAACGTTTAGCAAAGCTTTCCGGTGGTGTTGCTGTA	Canine
AGGAAAAGCTTAGAGAACGTTTAGCGAAGCTTTCCGGTGGCGTTGCTGTA	*E. equi*
AAGAAAAGCTACAAGAGCGTTTAGCAAAGCTTTCTGGTGGTGTTGCTGTA	*C. rum*

```
701
CTCAAGGTTGGTGGATCCAGCGAAGTTGAGGTGAAGGAACGCAAAGACAG      Ehr/8
CTCAAGGTTGGTGGATCCAGCGAAGTTGAGGTGAAGGAACGCAAAGACAG      FG1
CTCAAGGTTGGTGGATCCAGCGAAGTTGAGGTGAAGGAACGCAAAGACAG      FG2
CTCAAGGTTGGTGGATCCAGCGAAGTTGAGGTGAAGGAACGCAAAGACAG      OS
CTCAAGGTTGGTGGATCCAGCGAAGTTGAGGTGAAGGAACGCAAAGACAG      Harris
CTCAAGGTTGGTGGATCCAGCGAAGTTGAGGTGAAGGAACGCAAAGACAG      C8
CTCAAGGTTGGTGGATCCAGCGAAGTTGAGGTGAAGGAACGCAAAGACAG      Perth
CTCAAGGTTGGTGGATCCAGCGAAGTTGAGGTGAAGGAACGCAAAGACAG      HGE[a]
CTCAAGGTTGGTGGATCCAGCGAAGTTGAGGTGAAGGAACGCAAAGACAG      HGE[b]
CTCAAGGTTGGTGGATCCAGCGAAGTTGAGGTGAAGGAACGCAAAGACAG      HGE[c]
CTCAAGGTTGGTGGATCCAGCGAAGTTGAGGTGAAGGAACGCAAAGACAG      SH
CTCAAGGTTGGTGGATCCAGCGAAGTTGAGGTGAAGGAACGCAAAGACAG      SwH
CTCAAGGTTGGTGGATCCAGCGAAGTTGAGGTGAACGAACGCAAAGACAG      Canine
CTCAAGGTTGGTGGATCCAGCGAAGTTGAGGTGAAGGAACGCAAAGACAG      E. equi
TTGAAAGTAGGTGGATCTAGTGAAGTTGAAGTTAAGGAACGTAAAGATCG      C. rum

751
AGTAGAAGATGCGTTACATGCTA                                  Ehr/8
AGTAGAAGATGCGTTACATGCTA                                  FG1
AGTAGAAGATGCGTTACATGCTA                                  FG2
AGTAGAAGATGCGTTACATGCTA                                  OS
AGTAGAAGATGCGTTACATGCTA                                  Harris
AGTAGAAGATGCGTTACATGCTA                                  C8
AGTAGAAGATGCGTTACATGCTA                                  Perth
AGTAGAAGATGCTTTACATGCTA                                  HGE[a]
AGTAGAAGATGCATTACATGCTA                                  HGE[b]
AGTAGAAGATGCGTTACAT----                                  HGE[c]
AGTAGAAGATGCGTTACATGCTA                                  SH
AGTAGAAGATGCGTTACATGCTA                                  SwH
AGTAT----------------                                   Canine
AGTAGAAGATGCATTACATGCTA                                  E. equi
TGTAGAAGATGCTCTTCATGCAA                                  C. rum
```

Location of primers for the canine sample is highlighted in bold type

-	Data not available
FG	Feral Goat
OS	Old Sourhope
HGE[a]	Human granulocytic *Ehrlichia*; GenBank Accession No. U72628
HGE[b]	Human granulocytic *Ehrlichia*; GenBank Accession No. U96728
HGE[c]	Human granulocytic *Ehrlichia*; GenBank Accession No. AF033101
SH	Scottish horse
SwH	Swiss horse
C. rum	*Cowdria ruminantium*
Canine	Canine sample from Utrecht

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Natural prevalence of infection with *Ehrlichia* (*Cytoecetes*) *phagocytophila* of *Ixodes ricinus* ticks in Scotland

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Abstract

Ixodes ricinus nymphs and adults were collected from vegetation and from sheep at four sites in Scotland typical of areas endemic for tick-borne fever in sheep caused by infection with *Ehrlichia* (*Cytoecetes*) *phagocytophila* (Rickettsiales). The great majority of ticks examined was from woodland sites adjacent to sheep farms where there was a high probability of them feeding on roe deer (*Capreolus capreolus*) in a non-domestic focus of infestation and infection. Ticks were examined for infection by five methods. Batches of ticks were examined either by feeding on susceptible sheep or by feeding on rabbits and then prepared as stabilate which was inoculated into susceptible sheep. The sheep were monitored for clinical signs of tick borne fever. Batches of ticks were examined by polymerase chain reaction for *Ehrlichia phagocytophila*. Salivary glands were dissected out and stained by the Feulgen method to detect *Ehrlichia* masses, and were examined by indirect fluorescent antibody test. Each of the methods detected infection in ticks and the prevalence of infection in nymphs with the various methods ranged from >0.25% to 2.0%. Small samples of adults examined by Feulgen staining of salivary glands indicated infection prevalences of 2.1% in males and 1.6% in females. It is considered that these low infection prevalences may be typical of natural foci of infection where deer could be a major host of ticks and *E. phagocytophila*.

Keywords: *Ehrlichia phagocytophila*; *Ixodes ricinus*; Epidemiology; Rickettsia

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1. Introduction

Ehrlichia (*Cytoecetes*) *phagocytophila* is an obligate intracellular bacteria which causes tick-borne fever (TBF) in sheep and pasture-fever in cattle and is transmitted by *Ixodes ricinus* ticks (MacLeod and Gordon, 1933). Only nymphs and adult ticks are considered to be vectors for *E. phagocytophila* since transovarial transmission was found not to occur (MacLeod, 1936). The disease is characterised by high fever and severe leukopenia that may facilitate other infections like tick pyaemia, listeriosis, or louping-ill. Very young lambs are more resistant to signs of clinical infection (Stuen, 1993) although they undergo febrile symptoms and parasitaemia. After patent parasitaemia animals become carriers (Stuen, 1993) and that may be responsible for the maintenance of infection in the flock until the next generation of fully susceptible lambs arrives. Little is known about how efficiently ticks acquire and maintain infection in sheep populations or whether carrier sheep are in fact responsible for the maintenance of infection in the flock.

Ehrlichia phagocytophila survives for months in sheep; it can be detected in blood smears after splenectomy at 48 weeks (Foggie, 1951) or even 2 years after primary infection (Stuen, 1993). It is believed that the organism persists in blood, mesenteric glands, spleen and central nervous system (Gordon et al., 1932). It does not seem to survive in cattle as long as in sheep (Foggie and Allison, 1960). High levels of IgM are detected in carrier sheep (Scott, 1984) indicating continuous exposure of the immune system with the pathogen.

There is an increased interest in the study of the disease due to the recent diagnosis of a granulocytic species, genetically closely related to the ruminant isolates, affecting humans, dogs, and horses in Europe (Johansson et al., 1995; Clark et al., 1996), and the US (Chen et al., 1994; Greig et al., 1996). The close antigenic and genetic relationship raises the awareness of the zoonotic potential of *E. phagocytophila*-like organisms. It is also believed that the newly identified granulocytic isolates could be strain variations of *E. phagocytophila*. Although the vector for the newly diagnosed human granulocytic *Ehrlichia* has not been identified yet, it is suspected to belong to the genus *Ixodes* (Pancholi et al., 1995). The potential role of deer as reservoir of *Ehrlichia* species has been proposed (Foggie and Allison, 1960; Dawson et al., 1994). Wild rodents could play a role since they have been incriminated in the transmission of granulocytic ehrlichiosis in North America (Telford et al., 1996).

The aim of this study was to determine the prevalence of infection in vector *Ixodes ricinus* ticks by using various methods to confirm the results. Ticks for the study came from presumed sites of natural infection in deer adjacent to known foci of TBF in sheep. Ticks were collected predominantly from vegetation because this precludes the complication with feeding ticks which may contain infected fresh blood. Woodland sites inhabited by deer were predominantly sampled for ticks because of the much higher abundance of ticks there in comparison to sheep pastures.

A knowledge of the transmission rate could improve control of TBF in sheep by managerial maintenance of endemic stability. The infection prevalence in ticks is an essential characteristic in the overall transmission rate to sheep but little is known about the prevalence of *E. phagocytophila* in field ticks. The existing estimates of prevalence of

infection in ticks are high (MacLeod, 1936; Webster and Mitchell, 1989). Those figures need corroboration with different and newly developed methods.

2. Materials and methods

2.1. Tick samples

Questing ticks were collected from vegetation by blanket dragging in standardised transects on 20 occasions during the period from March to September in 1996 and 1997. Preserved sub-samples were identified using the key in Hillyard (1996). Four sites were used; Nos. 1–3 in Southwest Scotland and No. 4 in Southeast Scotland. Each site was characterised as a sheep farm on upland grazing bordering onto woodland. At each site roe deer (*Capreolus capreolus*) were present in the woods and at Site 1 there were in addition red deer (*Cervus elaphus*). The deer sometimes grazed on the sheep pasture, and rarely did sheep gain access to the woods. Samples were collected from both types of wood, from the sheep pastures, and on one occasion directly from sheep at Site 4.

2.2. Examination of tick salivary glands

Salivary glands of nymphs and adults were removed by dissection from the whole live ticks when stuck to wax and immersed in saline. Guts were removed from the ticks, then the glands were removed and placed in a drop of saline with 1% serum albumin on a microscope slide. Nymph salivary glands were removed together with the mass of remaining viscera. The liquid drop was spread and the glands teased into the film of liquid. The slides were dried then fixed for 1 h in 10% formalin. Glands were stained by the Feulgen method (Petrov, 1948; Bancroft and Stevens, 1982), which is specific for DNA. One batch of 50 ticks had been fed on a rabbit for 2 days to stimulate maturation of the *Ehrlichia*; the rest were from unfed ticks without any treatment.

2.3. Detection of infection in tick salivary glands by IFAT

Indirect fluorescent antibody test (IFAT) followed that of Paxton and Scott (1989). Dissected pairs of tick salivary glands on microscope slides were used as antigen. Glands were fixed for 15 min in acetone. A well was made around the glands with a paint pen. Non-specific reactions were blocked with normal rabbit serum at a 1/20 dilution in PBS (phosphate buffered saline, pH 8.0) for 30 min. Slides were washed three times in PBS. Sheep antiserum at 1:10000 titre against *E. phagocytophila* was diluted to 1/100 and 50 μl added to each well. Slides were incubated for 1 h, washed three times in PBS, then 50 μl of a donkey anti-sheep fluorescein isothiocyanate anti-IgG (whole molecule) conjugate (Sigma) was added at 1/160 dilution. Slides were incubated for 45 min then washed, dried and mounted in glycerol. After the IFAT coverslips and glycerol were washed off, the glands were fixed in formalin and stained with Feulgen to cross-check any glands positive by IFAT.

2.4. Detection of infection by polymerase chain reaction

Ixodes ricinus nymphs from vegetation were fed on rabbit ears for 2 days to stimulate maturation of any pathogen (Piesman and Spielman, 1982). Fed ticks were washed in water then placed in 1.5 ml Eppendorf tubes in batches of 10. Ticks were overlain with 50 μl of TE buffer (10 mM Tris, 1 mM EDTA, and 1% Tween 20) and crushed with the pipette tips. The samples were incubated at 55°C for 3 h with 0.5 μl of proteinase K (Sigma, 20 mg/ml) per sample then boiled for 10 min to inactivate the enzyme. The samples were clarified by centrifugation. To the supernatant liquid four volumes of absolute ice cold ethanol then 100 μl of 3 M sodium acetate were added. The samples were stored in a freezer for 48 h then the precipitated DNA was removed by centrifugation, washed twice in ice-cold 70% ethanol, then resuspended in distilled water. In addition, DNA from tick stabilates and a batch of 50 dissected salivary glands from 2-day fed nymphs was extracted using a QIAamp tissue kit (QIAGEN Ltd.) following manufacturers instructions.

Sixteen batches of 10 ticks each from two different sites, a pool of 50 tick salivary glands, and 8 tick stabilates were examined. Polymerase chain reaction (PCR) conditions and primer sequences were based on Munderloh et al. (1996). For the outer amplification primers 856 (5′-GAATTGACGGGGACCCGCACAAGCG-3′) and 1154 (5′-AAGGGCCGTGCTGACTTGACATCATC-3′) were derived from DNA sequences coding for *E. phagocytophila* 16S rRNA deposited in Genbank database. For inner amplification GER3–GER4 primers were used. For one group of samples a second round of amplification was done using 5 μl of the template and an annealing temperature of 60°C. 2 μl of loading buffer (75 mM EDTA, 0.1% bromophenol blue, 25% glycerol) were mixed with 10 μl of the PCR products and electrophoresed through 2% agarose gels at 70 V in TBE buffer (89 mM tris-borate, 2.5 mM EDTA) for 20 min. Gels were stained with ethidium bromide.

2.5. Determination of infection in ticks by exposure to sheep

Questing nymphs were examined by feeding on sheep which had been purchased from farms free of tick borne fever. Nymphs were enclosed on the sheep in cloth bags glued to the ears, and fed to repletion. Alternatively stabilates were prepared by feeding nymphs on rabbits for 2 days to stimulate maturation of any *Ehrlichia* present (MacLeod, 1936). Nymphs were removed, washed then suspended in a medium used for maintenance of *Cowdria ruminatium* (RPMI medium supplemented with 10% foetal bovine serum, 10% tryptose phosphate broth, 20 mM HEPES, 2 mM L-glutamine, 100 μg/ml of penicillin, 100 μg/ml of streptomycin, and 2.5 μg/ml of fungizone). Nymphs were ground briefly in glass tissue grinders and then filtered using a glass-fibre pre-filter in a re-usable 25 mm diameter filter holder. Dimethylsulphoxide (DMSO) was added to a final dilution of 10% v/v and the stabilate was stored over liquid nitrogen. Thawed stabilates were diluted 1:1 with PBS then inoculated intravenously at a dose of 50 nymph equivalents. The sheep were monitored daily for infection over 14 days post inoculation by observation of behaviour, rectal temperature and blood smears stained with Giemsa's stain. A minimum of 100 neutrophils were examined by microscopy to determine percentage of neutrophils

infected with *Ehrlichia* morulae. Sera were tested using the IFAT of Paxton and Scott (1989).

2.6. Transmission of Ehrlichia from sheep to sheep via ticks

To provide additional confirmation of the identity of the agent isolated from ticks a blood stabilate was made from a ewe sheep infected following feeding of field ticks. Venous blood was collected during patent parasitaemia and DMSO was added to a final volume of 10% before storage over liquid nitrogen. This stabilate was inoculated intravenously to infect a susceptible ewe. Larval *I.ricinus*, which had been derived from feeding females on rabbits, were applied in ear bags to this ewe when it had patent parasitaemia. The resulting nymphs were fed in a batch of 200 on another susceptible ewe which was monitored for infection as described above.

2.7. Calculation of infection prevalence from batches of ticks

To calculate the infection prevalence of ticks when they were examined in batches the formula of Chiang and Reeves (1962), as described for batches of ticks by Norval et al. (1990), was used.

3. Results

3.1. Tick collections

No ticks other than *Ixodes ricinus* were found. The great majority was found in the woodlands and during the April–May peak of activity. Tick numbers on sheep pasture were very low for equivalent sampling effort and only 20 of these nymphs were tested.

3.2. Salivary gland infections assessed by Feulgen staining

A total of 554 ticks were examined by Feulgen staining. The results are shown in Table 1 and a preparation is shown in Fig. 1. The overall infection prevalences shown by Feulgen staining of salivary glands was 1.4% in nymphs, 1.6% in females, and 2.1% in males. All positive ticks were collected from woodland. Table 2 shows the intensity of infection of the ticks, in most of the ticks considered infected the salivary glands were dominated by the masses of presumed *Ehrlichia*, with most salivary acini infected with one or more masses. Type 1 acini were not seen infected. The masses were large relative to the host cell and stained densely and conspicuously in the unfed ticks. Many of the *Ehrlichia* masses in the one positive salivary gland (Fig. 1) from the nymphs fed for 2 days were substantially enlarged relative to those seen in unfed nymphs, but no hypertrophy of the host cell nucleus was seen.

3.3. Salivary gland infection assessed by IFAT and Feulgen staining

One of the batch of 111 nymph salivary glands tested by IFAT was positive and the fluorescent masses had morphology similar to presumed *Ehrlichia* stained with Feulgen. A negative gland in Fig. 2 is compared to a positive gland in Fig. 3. When these glands

Table 1
Infection of ticks according to instar and location determined by Feulgen staining of salivary glands

Site	Instar	No. examined	No. positive
1	Nymphs	249	5
	Males	34	1
	Females	26	1
2	Nymphs	105	0
	Males	9	0
	Females	21	0
3	Nymphs	79	1
	Males	5	0
	Females	6	0
4	Nymphs	12	0
	Females	8	0

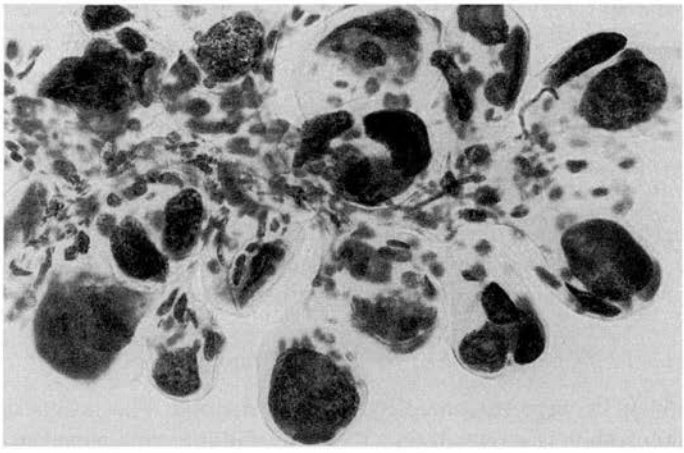

Fig. 1. Portion of a salivary gland of a nymph of *Ixodes ricinus* fed for 2 days and stained by the Feulgen method; showing many large densely staining masses of presumed *Ehrlichia*, and acini enlarged by feeding.

were subsequently stained by Feulgen none of the remaining 81 was positive. The other 30 pairs of glands were not retained on the slides during processing, including the one positive by IFAT.

Table 2
Numbers of *Ehrlichia* masses per infected tick (intensity of infection)

	No. positive	No. masses
Nymphs	5	19, 64, 88, 90, 130, 140
Female	1	384
Male	1	20

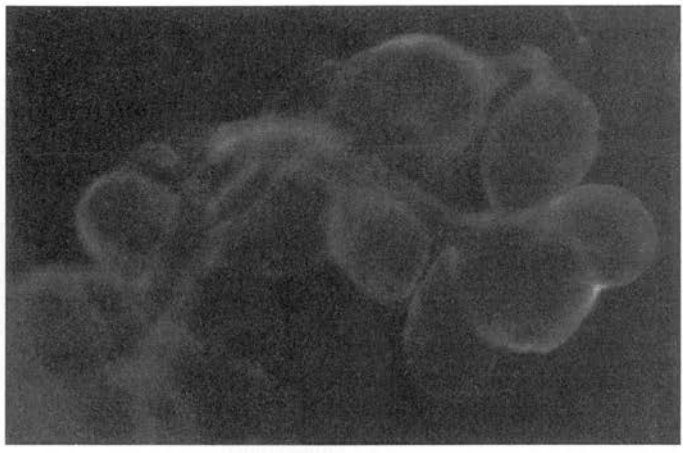

Fig. 2. Portion of an uninfected salivary gland of a nymph of *Ixodes ricinus* stained by IFAT to detect *E. phagocytophila*; no positive staining.

3.4. Polymerase chain reaction

One of the 16 batches of 10 fed ticks was positive. The prevalence of infection was calculated to be 0.64%. The single batch of 50 salivary glands from nymphs which had been pre-fed for 2 days was positive, indicating at least one out of 50 (2%) of these ticks was positive. One of the eight batches of 50 nymphs fed for 2 days and prepared as stabilate for inoculation into sheep was positive, and the calculated prevalence was 0.27%. Fig. 4 shows typical results. After further amplification of a 5 μl aliquot of the

Fig. 3. Portion of an infected salivary gland of a nymphs of *Ixodes ricinus* stained by IFAT to detect *E. phagocytophila*; three positive bodies shown.

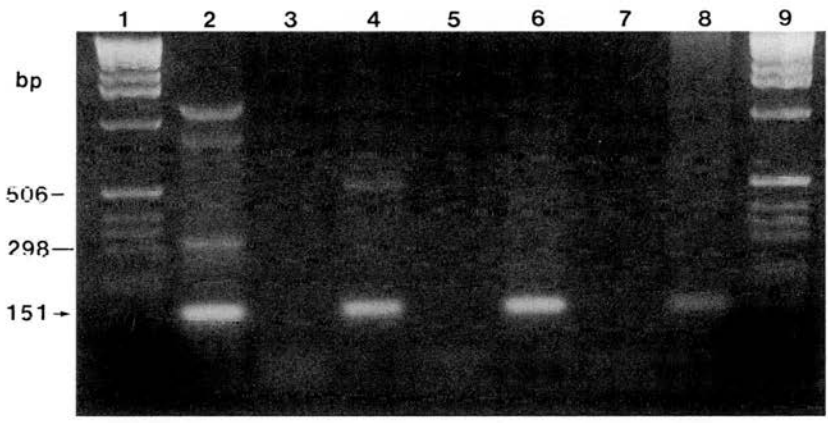

Fig. 4. Agarose gel (2%) of PCR products using GER3–GER4 primers in the nested reaction to amplify DNA of the granulocytic *Ehrlichia* in the *E. phagocytophila* genogroup. Lanes 1 and 9 contain molecular size markers, 1 Kb ladder. PCRs were performed with DNA extracted from the following samples: *E. phagocytophila* (feral goat) blood stabilate (lane 2) as the positive control; water (lane 3) as the negative control; lanes 4 and 5 represent two batches of 10 nymphs of *Ixodes ricinus* fed for 2 days; lanes 6 and 7 represent two batches of 50 *Ixodes ricinus* nymphs fed for 2 days and prepared as stabilates; lane 8 represents one batch of 50 salivary glands dissected from 2-day-fed nymphs of *Ixodes ricinus*. Lanes 4, 6, and 8 show the expected PCR product at 151 bp. Gel was stained with ethidium bromide and illuminated by uv light.

PCR product again using the internal pair of primers (GER3–GER4), each of the eight stabilates gave a positive result.

3.5. Transmission experiments

The transmission experiments with live ticks and tick stabilates are summarised in Tables 3 and 4. The calculated infection prevalence of the batches of ticks exposed to sheep 1–4 was 0.47% and that of the batches of ticks exposed to sheep 5–12 was 0.57%.

Clinical TBF was produced in a susceptible ewe by feeding on it nymphs which had been fed as larvae on an infected ewe. The temperature exceeded 41.0°C on day 4 post-inoculation, and pyrexia lasting until day 9. *Ehrlichia* morulae in neutrophils were first seen on day 4 and reached a maximum parasitaemia of 29% on day 6. Post-infection sera was positive by IFAT for *E. phagocytophila* at 1/640 dilution.

4. Discussion

A low prevalence of infection was detected in these ticks. Our results are in agreement with the infection prevalences detected in ticks from California (Barlough et al., 1997) but not with the data from the eastern and midwestern United States (Magnarelli et al., 1995) or a previous study in Scotland (Webster and Mitchell, 1989). Our results are from field ticks collected largely in woodland sites and may relate to foci of natural infection in

Table 3
Summary of transmission experiments from field ticks to sheep by feeding or inoculation of stabilates

Sheep no.	Ticks	Site	Fed/stabilate	Infection
1	200 N [a]	1+2	Stabilate	+
2	75 N	3	Stabilate	−
3	100 N, 18 F [b]	1+2	Fed	−
4	200 N	1	Fed	+
5	50 N	1	Stabilate	−
6	50 N	1	Stabilate	+
7	50 N	1	Stabilate	−
8	50 N	1	Stabilate	−
9	50 N	1	Stabilate	−
10	50 N	1	Stabilate	+
11	50 N	1	Stabilate	−
12	50 N	1	Stabilate	−

[a] N, Nymphs.
[b] F, Females.

deer, whereas ticks in the latter study were infected by feeding in experimental sheep and tested by antibody coated electron microscopy grids.

Feulgen staining was the simplest of the various methods of assessing infection in ticks and also, it gave data on intensity of infection. It has the great advantage of indicating infection that is likely to be competent for transmission at the next feed because the *Ehrlichia* has been developed in cells of the salivary glands. However, it is not specific for *Ehrlichia*. Feulgen staining will also detect *Babesia* sporoblasts in salivary glands (Petrov, 1948) but these sporoblasts are very large and additionally induce massive hypertrophy of the acinar host cell nucleus. The IFAT can be used in conjunction with Feulgen staining in order to determine the identity of infection with granulocytic *Ehrlichia*. PCR specifically identified the ticks as truly infected with *Ehrlichia phagocytophila*-like organisms. Due to the low infection prevalence this complex method is not ideal for routine epidemiological survey using individual ticks in these localities and should be used, in conjunction with Feulgen staining to verify the identity of the *Ehrlichia* in batches of ticks. PCR may have sensitivity problems producing false negatives depending on DNA extraction procedures, or Taq enzyme inhibitors. Misleading positives may be produced by PCR used at high sensitivity in repeated cycles of amplification because of the tendency for vector borne pathogens to enter many individual vectors in high numbers but then be killed by the defences of a high proportion

Table 4
Clinical criteria of infection in sheep

Sheep No.	T>41.0 at day	1st day parasitaemia	Max. % parasitaemia	T<41.0 at day
1	6	5	25	11
4	5	6	14	9
5	6	6	21	12
9	6	7	10	13

of the vector population. Residual DNA from the killed pathogens or from live pathogen in a location in the vector unsuitable for transmission or in too small amount to be infective, may be detected by PCR when used to extremes of sensitivity. This may explain the discrepancy between our results with the eight batches of 50 ticks tested by inoculation into sheep, and by low sensitivity and high sensitivity PCR. Information on the control of TBF in sheep often implies that all *Ixodes ricinus* on sheep pastures in endemic areas are likely to be infected (Henderson, 1990). Our data are not consistent with this premise but tick populations supported by sheep may have higher prevalences of infection and this needs to be investigated further.

Adult ticks are expected to have higher prevalences of infection because larvae and nymphs maintain infection even after feeding on refractory hosts (Webster and Mitchell, 1989). Our results from small samples of adults corroborate this finding. Field infestation of deer and sheep with nymphs and adults at the time of peak tick activity (April–May) at these sites would exceed tens of nymphs attaching per day, and at least one female per day (Milne, 1948). Thus exposure of every animal to *E. phagocytophila* remains likely.

Nymph and adult *Ixodes ricinus* can survive for at least 1 year (MacLeod, 1932), and therefore are capable of being a long term reservoir of infection. Transmission experiments performed by MacLeod (1936) lead to the conclusion that the carrier state was infective to ticks. Therefore, it would be expected that tick infection prevalence should be high but our results are not compatible with this. It is of great epidemiological importance to verify the role of carrier infections in sheep and deer in the maintenance of infection. Similar estimations of infection prevalence in field ticks are also needed for risk assessment of diseases of potential public health importance, such as Lyme borreliosis, human granulocytic ehrlichiosis and babesiosis.

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